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THE BIOCHEMICAL JOURNAL

EDITED FOR THE BIOCHEMICAL SOCIETY

BY

HAROLD WARD DUDLEY

AND

ARTHUR HARDEN

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OBITUARY NOTICES.

EGERTON CHARLES GREY (1887–1928).

EGERTON CHARLES GREY was the second son of the late Colonel Arthur Grey. His earlier years were spent in Paris, after which he went to Australia and graduated in the University of Sydney. Here he carried out some experiments on the fatty acids of the human brain. In 1912 he came to England with an 1851 Exhibition Scholarship and commenced at the Lister Institute the work on the chemical action of bacteria which he continued to pursue until his death. He subsequently became a Beit Fellow and a John Foulerton Student of the Royal Society, working for some time at the Pasteur Institute, where he acquired a great admiration for the work of Pasteur, and at the Biochemical Laboratory, Cambridge. During this time he was awarded the degrees of M.A. (Cantab.) and D.Sc. (London) and completed the work for his medical qualification, which he had commenced in Sydney.

During the war he served as second lieutenant in the Royal Fusiliers, and was wounded at Gallipoli and invalided from the service. He subsequently entered the Navy and served as Surgeon-sub-lieutenant to H.M.S. *Nereide* and as interpreter in French and German, taking part in the operations in the Black Sea. After the war Grey was appointed Professor of Chemistry in the University of Cairo, and for his services in this capacity was decorated with the Order of the Nile. His experiences here led him to write a short book, *Practical Chemistry by Micro-Methods* (Cambridge and London, 1925), in which a method of teaching analytical chemistry with the aid of a minimum amount of apparatus and reagents is described.

In 1926 he relinquished his appointment at Cairo and undertook an investigation of the food problems of Japan on behalf of the League of Nations. He spent about six months in Japan and made, single-handed, analyses of all the chief foodstuffs of the country. The results form the subject of a report issued by the League of Nations and are also contained in a series of articles in the Japanese weekly review, *The Trans-Pacific* (July and August, 1927). He returned to work at Cambridge, where he died after a very short illness on August 10th, 1928.

Grey was a man of attractive but unconventional character, full of enthusiasm for his subject. His early death is a great loss both to his friends and to biochemistry.

Grey's experiments on the chemical action of *B. coli communis* commenced at a time when Penfold had just shown that by cultivation in a medium containing sodium chloroacetate, a strain of this organism could be isolated

which did not produce gas from glucose. The nature of the change thus produced in the enzymic armoury of the organism formed his first subject of investigation and led him to the idea that two (or more) independent sets of enzymes were active in this fermentation. He developed this idea by studying the progressive changes during fermentation. By using a large inoculation and by avoiding, as far as possible, the complications due to the growth of the organism he was able to distinguish three characteristic periods. Immediately following inoculation many of the organisms die and the products formed from glucose are mainly alcohol, acetic acid, succinic acid and formic acid. During the next period the production of these ceases, some of them are even decomposed, whilst part of the sugar is synthesised into a non-reducing carbohydrate and another part converted almost wholly into lactic acid. At the same time the surviving organisms begin to multiply. Finally the synthesised carbohydrate is hydrolysed and fermented along with the remaining sugar, both lactic acid and the alcohol group of products being formed. Another series of observations was made on the influence of aerobic or anaerobic cultivation on the subsequent behaviour of the organism to sugar. When the immediate past history of the organism has been anaerobic, the fermentation under anaerobic conditions yields very little or no lactic acid and much less succinic acid than when its conditions of cultivation have been aerobic. These observations led Grey to the conclusion, expressed in a paper which was in the Press at the time of his death, that *B. coli* can decompose sugar in at least two different ways, by a lactic fermentation and by a modified alcoholic fermentation. The latter of these is only possible when the organism has been recently grown in the presence of free oxygen. Generalising from this he concluded that alcoholic fermentation is due to the continued action under anaerobic conditions of an enzyme, zymase, which is the surviving portion of the respiratory mechanism of the organism. Whether or not these ideas are accepted, they bear witness to the breadth of vision which was characteristic of their author and provide ample ground for further investigation.

A. H.

DIARMID NOËL PATON (1859–1928).

THE death of Diarmid Noël Paton in his 70th year on September 30th, 1928, on the day on which his resignation of the Regius Chair of Physiology in Glasgow University took effect, has removed one of the earliest research workers in the field of chemical physiology in this country. So long ago as 1884 he began an investigation on the subject of urea formation and to the end of his life he was keenly interested in all metabolic problems. His interests were not confined to an interpretation of chemical physiology in any narrow sense. He was an investigator in many other physiological fields. He worked on the endocrine glands long before they became a popular subject of research; he directed investigations into dietary problems, particularly of the poor, and, later, into the relation of nutrition to the incidence of rickets and of growth. His last work was on postural reflexes and postural apnoea in the duck and the swan.

He was a splendid teacher, expressing his views clearly and succinctly. Although he held strong opinions on many problems he was scrupulous to put before his students all aspects of the question. He demanded that his students should use their intelligences and make up their own minds. *Ex cathedra* utterances were anathema to him. He remembered, too, that the majority of his auditors were going to be ordinary practitioners of medicine. He was well equipped for stressing the practical aspect of physiology looking back, as he did, to his own early days when he was a Clinical Tutor at the Edinburgh Royal Infirmary. He regarded and taught physiology as a whole, not as a variegated pattern of specialist problems put together for narrow and self-contained specialist workers.

Although he did not suffer fools gladly, and although he was perhaps sometimes rather excessively intolerant, hidden within his rather formal but graceful exterior was a most warm and kindly heart. He was always ready to help to the full extent of his powers anyone in real distress or difficulty. He had a shy sensitive nature which yearned for friendship.

As a research worker Noël Paton was keen, energetic, critical and thorough. He hated all cheap and shoddy work and, above all, he loathed special pleading, specious reasoning and the suppression of data which did not fit the hypothesis put forward. He tried to play the game himself and he expected his antagonists to have his own high sense of honour. He lived for science; science was indeed a religion to him and he could not tolerate any desecration by insincere or half-hearted self-advertising work.

E. P. C.

I. THE CHEMICAL ACTION OF QUINONES ON PROTEINS AND AMINO-ACIDS. PART II.

By EVELYN ASHLEY COOPER and RAYMOND BENNETT HAINES.

From the Chemical Department, University of Birmingham.

(*Received November 15th, 1928.*)

It was concluded from previous experimental work [Cooper and Haines, 1928, 1] that the bactericidal action of the quinones was associated with their chemical action on the simpler cell-constituents, such as the amino-acids, rather than with their chemical reactivity with the complex colloids, *e.g.* proteins. Subsequently, however, it was found [Cooper and Haines, 1928, 2] that the temperature-coefficient for the reaction between benzoquinone and glycine was the normal one for a chemical process, yet quinone was twenty times more active as a germicide at 37° than at 20°. It was then found however that this considerable accession in bactericidal power with rise in temperature was not restricted to quinone, but was a common property of oxidising disinfectants. It would appear thus that the main factor in the germicidal action of the quinones is the interaction with the amino-acids, but that another factor fundamental in the action of oxidising disinfectants is operating simultaneously.

The earlier work had shown that there was a general correspondence in the relative germicidal power of benzoquinone and toluquinone and their chemical activity with various amino-acids. Benzoquinone was found to react from four to five times as rapidly with amino-acids as toluquinone, while the ratio of their bactericidal action varied on an average from five to seven (and sometimes higher). It was also found that the presence of salts increased the velocity of reaction with glycine. Further experiments have therefore been carried out to ascertain the relative effect of salts on reaction-velocity in the case of both benzo- and tolu-quinone, as, if it could be shown that the inorganic constituents of living cells increased the reactivity of benzoquinone more than that of toluquinone, some explanation of the foregoing discrepancy would be offered.

0·2 % solutions of the two quinones were prepared, (1) in water, and (2) in Ringer's solution (the bicarbonate being excluded). 0·5 g. of glycine was then dissolved in 100 cc. of each solution, and the amount remaining in solution at stated times was determined by withdrawing 5 cc. and titrating by Valeur's method (Table I).

Table I. % *remaining in solution at* 20°.

(*Benzoquinone.*)

Time min.)	(1) Water	Time (min.)	(2) Ringer's solution
0	100	0	100
10	98·7	10	93·7
30	96·6	35	90·8
70	90·4	100	86·0
120	87·2	160	81·2
235	83·5	240	74·9
360	74·5	360	65·2

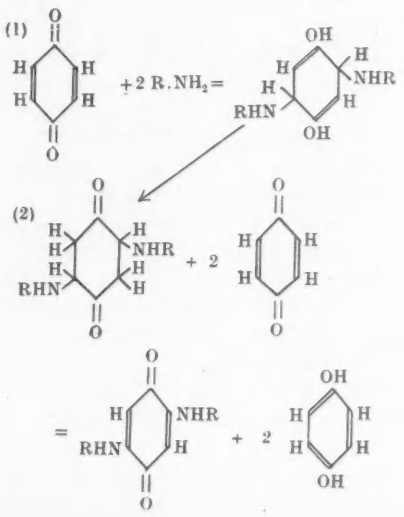
(*Toluquinone.*)

0	100	0	100
20	98·2	10	99·5
60	96·4	35	96·4
120	95·2	60	94·8
180	92·3	165	92·1
240	91·1	240	90·5

The saline constituents of Ringer's solution thus considerably accelerate the reaction between benzoquinone and glycine, but have only a slight effect with toluquinone. The inorganic salts present in the cell may thus be supposed to raise the reaction-velocity of benzoquinone, and the great difference in the germicidal power of benzo- and tolu-quinone is explicable.

Apart, however, from velocity considerations, it will be shown that benzoquinone is capable of effecting more cell-destruction than toluquinone per gram-molecule.

Ville and Astre [1895] and Suchanek [1914] showed that the interaction of quinones and amino-compounds took place in several stages as follows:



The addition product can be isolated from either benzene or ether solution; in water or alcohol, however, the reaction continues by oxidation to the formation of the substituted quinone and quinol.

In the case of benzoquinone a dianilido-compound is formed, two nitrogen atoms being introduced. With toluquinone, although a similar addition compound is formed intermediately, the final derivative contains only one nitrogen atom. Thus three molecules of benzoquinone react with two of amino-compound, but two molecules of toluquinone react with only one of the amino-substance.

Xyloquinone forms an addition product, but is incapable of yielding a final substituted quinone, the reaction ceasing at the first stage.

Chloroquinones on the other hand [Astre, 1897] add on two molecules of amino-compound, and both nitrogen atoms finally enter the nucleus with evolution of hydrochloric acid. Chloroquinones are thus as effective as benzoquinone in taking the reaction completely to the oxidation stage.

These observations are analogous to those already made in the case of bactericidal action [Cooper and Haines, 1928, 1]. The homologues are less active as germicides than benzoquinone itself, whilst *m*-dichloroquinone is as efficacious as the latter.

Furthermore, alcohol facilitates the completion of the reaction to the oxidation stage, and also increases the germicidal power of the quinones.

The fact that three molecules of benzoquinone react with two of amino-acid, whilst two molecules of toluquinone are required for the reaction with one molecule of the amino-acid, may also explain the observation already referred to that the difference in the germicidal powers of benzo- and toluquinone is somewhat greater than would be expected from their reaction-velocities with amino-acids. It is evident that per gram-molecule benzoquinone would have a greater destructive effect on the cell than toluquinone in the proportion 3/2.

In order to throw further light on this problem the additive and final oxidation products were prepared from benzoquinone and anthranilic acid, and their germicidal action was studied. The additive product was only germicidal in a concentration as high as 1 in 3000 towards *B. coli* (30 min. at 20°); under the same conditions benzoquinone is active in 1 in 35,000. The oxidation product was too insoluble for examination. Although it would seem that it is the final oxidation process that plays the chief rôle in the bactericidal action of the quinones, the fact that benzoquinone suffers such an extensive depreciation in germicidal power merely by forming an addition compound with anthranilic acid suggests that the bactericidal process is also bound up to a certain extent with the preliminary addition reaction.

Reference has already been made to the finding that the temperature-coefficient for the interaction of benzoquinone and glycine is the normal one for a chemical process, whilst at 37° the quinone is twenty times as powerful a germicide as at 20°.

The exact germicidal temperature-coefficient, however, had not been determined under the conditions employed, all that had been ascertained being the relative concentration required for disinfection in a fixed period at 20° and 37°.

The temperature-coefficient for bactericidal action was therefore next measured by means of a modified technique.

Selected concentrations of phenol and quinone were inoculated with a definite volume of culture at 20° and 37°, and by subculturing at stated intervals into nutrient broth the periods required for complete disinfection at the different temperatures could be ascertained. The ratio of the times is a measure of the temperature-coefficient of the process (Table II).

Table II.

	Period of disinfection (min.)		Temperature-coefficient per 10°
	20°	37°	
B. coli			
Phenol, 1 in 200	150	27·5	3·2
Quinone, 1 in 50,000	72·5	7·5	5·7
B. fluorescens			
Phenol, 1 in 200	67·5	12·5	2·9
Quinone, 1 in 80,000	42·5	< 4	>6

The observations confirm the original suppositions; the temperature-coefficient of disinfection in the case of quinone is 6 or even higher, the coefficient for the chemical action on glycine being the normal one (2 for a 10° rise). In the case of phenol the temperature-coefficient for germicidal action is lower (approximately 3) and agrees more closely with the coefficient for the process of protein-coagulation by phenol, viz. 2 [Cooper and Haines, 1928, 2].

m-Cresol, on the other hand, was found not to increase in germicidal power with rise in temperature, 1 in 500 concentration being equally bactericidal at 20° or 37°. It was of interest therefore to extend the protein coagulation experiments to the case of this homologue of phenol.

100 cc. of egg-white were made up to 200 cc. with distilled water and filtered; the filtrate was diluted with an equal volume of water and again filtered. 100 cc. of the final filtrate were mixed with 100 cc. of 1 % *m*-cresol solution in a large bottle and kept at constant temperature in a thermostat. The rate of coagulation was determined at 20° and 30°, 10 cc. of the mixture being withdrawn at intervals and rapidly filtered.

The amount of protein in the filtrates was then estimated by heat-coagulation and weighing (Fig. 1).

The results, plotted in Fig. 1, show that the velocity of coagulation by *m*-cresol is only very slightly increased by rise in temperature, and are thus consistent with the bactericidal observations. The physico-chemical explanation of the germicidal power of the phenols is thus quite in accordance with the facts.

In the case of quinone, however, the chemical interpretation already put forward, whilst entirely consistent with many observations, does not at the present time account for the high temperature-coefficient for germicidal action.

Table III. *% quinone taken up from the solution.*

Pathological source of urine	Benzoquinone		Toluquinone		Ratio	
	½ hr.	1 hr.	½ hr.	1 hr.	½ hr.	1 hr.
Leucaemia	7·7	18·6	3·6	8·4	2·1	2·2
,,	67·6	—	37·9	—	1·8	—
Nephritis	11·2	25·0	8·2	18·1	1·4	1·4
Jaundice	5·44	25·0	5·2	25·1	1·0	1·0
Haematuria	41·0	66·0	28·6	53·1	1·4	1·3
,,	58·4	—	40·6	—	1·4	—
Glycosuria	35·8	—	6·1	—	5·9	—
,,	6·8	—	1·3	—	5·2	—

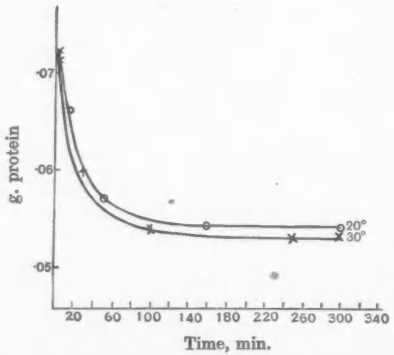


Fig. 1. Velocity of coagulation of albumin by *m*-cresol.

The possible application of the quinones in medicine.

It was previously shown [Cooper and Haines, 1928, 1] that alcoholic solutions of benzoquinone react rapidly with amino-acids, and since the nitrogenous constituents of urine vary considerably in health and disease it was thought that the amount of reaction might be of use as an index in diagnosis. Accordingly experimental work in this direction has been carried out. The clinical significance of the benzoquinone/toluquinone reactivity ratio has also been considered. In general, 10 cc. of the specimen of urine were allowed to react with an equal volume of 0·4 % solution of the quinones in 25 % alcohol for definite periods, the course of the reaction being followed by the volumetric estimation of the quinone remaining unused in the solution (Table III).

The chemical reactivity ratio of the two quinones is thus highest in the case of glycosuria (5·5), intermediate in value with leucaemia (2), and lowest in nephritis, jaundice and haematuria (1·4). It would thus seem that the deter-

mination of this ratio would afford a basis for a test which might be of value in medical diagnosis. The process resolves itself into a simple titration with thiosulphate under standard conditions, and can be carried out in 30 minutes to 1 hour.

The authors desire to express their best thanks to the Department of Scientific and Industrial Research for a maintenance grant which has made it possible to carry out this investigation.

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II. THE BACTERICIDAL ACTION OF THE NITROSO-COMPOUNDS.

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(*Received November 15th, 1928.*)

Earlier investigations [Cooper, Woodhouse, and Forstner, 1925; Cooper and Forstner, 1926] have shown that the nitrosoanilines are much more effective germicides than the corresponding nitrosophenols. Nitrosoaniline, for example, is inhibitory in concentrations of 1 in 100,000, whilst with nitrosophenol concentrations of 1 in 20,000 are necessary to inhibit bacteria. Notwithstanding this activity it is characteristic of both types of compounds that their high germicidal power is only apparent in long period bactericidal tests, such as the inhibitory method which covers a period of 48 hours. In the ordinary short methods of measuring germicidal power ($\frac{1}{2}$–3 hours) the nitroso-compounds reveal only a low efficiency.

The present investigation has been concerned with the two following problems.

1. The possible application of the nitrosoanilines as internal disinfectants.
2. The mechanism of their bactericidal activity.

1. *The nitrosoanilines as disinfectants.*

Nitrosoaniline and nitrosodimethylaniline have the disadvantage of being slightly soluble in water. The hydrochloride is readily soluble, but is much less efficacious. The methyl iodide addition product, on the other hand, is not very soluble, and is also much less effective than the free base.

The results in Table I illustrate these points.

Table I. *Inhibitory concentrations.*

B. coli, 48 hours at 37°

Nitrosodimethylaniline, 1 in 170,000
Nitrosodimethylaniline hydrochloride, 1 in 7,500
Nitrosodimethylaniline methiodide, >1 in 25,000

The loss in activity cannot be explained on the assumption of partition-changes between the phases within the cell caused by differences in solubility, as the readily soluble hydrochloride and the insoluble methiodide, which is, in fact, less soluble than the free base, are both weak germicides. It is concluded therefore that the fall in bactericidal power is associated with the change in

the valency of the amino-nitrogen to the quinquevalent state, and an essential condition for germicidal efficiency in the case of the nitrosoanilines is the presence of nitrogen in the tervalent state as in the amino- or substituted amino-group.

Nitrosodimethylaniline however is easily soluble in glycerol, propylamine, or formamide without significant loss in germicidal power, and the possibility of its clinical application is at present under investigation.

2. *The mechanism of bactericidal action.*

It has already been pointed out that the nitroso-compounds are slowly-acting germicides. In order to understand the nature of their bactericidal action, quantitative studies have been carried out to ascertain the reactivity of these substances towards amino-acids, proteins, lipins, and nucleic acids. These experiments involved the volumetric estimation of *p*-nitrosophenol and *p*-nitrosodimethylaniline in aqueous solution, and this was carried out by titration in acid solution with titanous chloride.

In general 30 cc. of the aqueous solution of the nitroso-compound was placed outside a viscose dialyser contained in a broad stoppered bottle. Inside the dialyser was placed 1 g. of the substance with which the solute was to react, *e.g.* protein, etc., either dissolved or suspended as the case might be in a measured volume of water. Equilibrium, *i.e.* maximum uptake of the nitroso-compound, was found in all cases to be complete within 72 hours. The initial and final concentrations being determined by analysis, the amount of nitroso-compound reacting or absorbed by 1 g. of the substance could be ascertained. The experiments were carried out at 20° (see Table II).

Table II. *Uptake of nitroso-compound* % (i.e. % *fall in concentration in aqueous phase*) *by* 1 *g.*

	(*a*) Gelatin	(*b*) Albumin	(*c*) Edestin	(*d*) Nucleic acid*
Nitrosophenol	4·9	3·5	4·2	9·7
Nitrosoaniline	5·0	—	0·0	46·0
Nitrosodimethylaniline	0·9	5·6	0·4	22·2

* The nucleic acid was first dissolved in dilute sodium hydroxide solution and the solution neutralised with acetic acid.

Consideration of the results shows that the nitroso-compounds are taken up in large amounts by nucleic acid, but only to a very small extent by proteins. Quantitative experiments showed that there was also no reaction at ordinary temperatures between nitroso-compounds and glycine, diamino-acids or a mixture of amino-acids from autolysed yeast. Furthermore, when solutions of nucleic acid and *p*-nitrosoaniline were mixed together and allowed to stand, a greenish black precipitate was gradually deposited, pointing to a chemical reaction between the two substances. The product was subjected to complete analysis with the following results:

$$C = 50{\cdot}8, \quad N = 14{\cdot}2, \quad H = 5{\cdot}6\ \%.$$

The nucleic acid from which the substance was prepared had the composition:

C = 32·8, N = 14·16, H = 5·1 %.

It is difficult to draw definite conclusions, but it is possible that the compound is a salt of the nitroso-base with the nucleic acid. The reaction between nitroso-compounds and nucleic acid proceeds with extreme slowness and is scarcely perceptible in a period of three hours, but becomes measurable in periods of from 24 to 72 hours.

There is thus a distinct analogy in the case of nitroso-compounds between the process of germicidal action and the reactivity with nucleic acid, an essential constituent of cell nuclear material and associated with the mechanism of growth. Such cell-constituents as proteins and amino-acids are not appreciably attacked by nitroso-compounds, and the bactericidal action of these slowly acting disinfectants would appear to depend on a gradual but effective interaction with the nuclear constituents. Phenols such as phenol, the cresols, dihydroxybenzenes, and chlorophenols have no precipitating action on the nucleic acids, their germicidal action being more associated with their denaturing action on proteins.

With the object of throwing further light on the problem the influence of concentration on the uptake of *p*-nitrosodimethylaniline by nucleic acid was studied by the dialysis method already described (Table III).

Table III. *Final concentration of* p-*nitrosodimethylaniline.*

(*a*) Nucleic acid g. per g.	(*b*) Water-phase g. per cc.	Distribution ratio *a*/*b*
0·01139	0·000797	14·3
0·01577	0·001078	14·5
0·02330	0·001594	14·5
0·02612	0·001885	13·8

The distribution ratio tends to approximate to a partition-coefficient, the base being as much as 14 times as soluble in nucleic acid as in water. The constancy of this ratio suggests that a considerable proportion of the base is merely dissolved in the nucleic acid and the amount in actual chemical union is relatively small.

In order that the *p*-nitrosodimethylaniline may gain access to the nuclear substance of the cells, ready solubility in the intervening cell-constituents is essential, and a series of experiments was therefore next carried out to determine the solubility of the base in a lipin, *e.g.* lecithin.

A stable lecithin emulsion was prepared by dissolving the lipin in ether and pouring the solution into excess of water, the ether then being removed by means of a current of air.

A 5 % emulsion could thus be prepared. The influence of concentration on the absorption of nitroso-compounds, and also of quinones and phenol, was investigated, the dialysing method being again employed (Table IV).

Table IV. *Final concentrations.*

(*a*) Lecithin g. per g.	(*b*) Water-phase g. per cc.	Distribution ratio *a*/*b*
Benzoquinone		
0·01101	0·005119	2·2
0·01061	0·004255	2·5
0·00901	0·003386	2·7
0·00793	0·002538	3·1
0·00619	0·001684	3·7
Toluquinone		
0·03060	0·005136	6·0
0·02487	0·004303	5·8
0·01592	0·003440	4·6
0·01194	0·002720	4·4
0·00448	0·001684	2·7
Nitrosophenol		
0·04106	0·003599	11·4
0·03367	0·003092	10·9
0·02590	0·002546	10·1
0·02122	0·001713	12·4
0·01246	0·001182	10·5
0·00646	0·000576	11·2
Nitrosodimethylaniline		
0·01778	0·001301	13·7
0·01119	0·000952	11·8
0·00901	0·000796	11·3
0·00659	0·000583	11·4
0·00428	0·000398	10·8
0·00165	0·000194	9·0
Phenol		
0·5141	0·05501	9·3
0·3758	0·04709	7·8
0·1821	0·03558	5·1
0·1741	0·02369	7·4
0·0910	0·01184	7·7
0·0396	0·00595	6·7

Inspection of the results indicates that the nitroso-compounds amongst the substances examined are most readily absorbed by the lecithin. In the case of *p*-nitrosophenol the distribution-ratio is almost constant over a wide range of concentration and the process is one of simple solution in the lipin, *p*-nitrosophenol being about 11 times as soluble in lecithin as in water.

The distribution-ratio of *p*-nitrosodimethylaniline increases somewhat with rise in concentration of 9·0–13·7, suggesting again a process of solution complicated by molecular association of the solute in the lipin. Similar results are obtained with phenol and toluquinone. With benzoquinone, however, the distribution-ratio falls with increasing concentration, suggesting that adsorption or surface-concentration predominates (Fig. 1). The constancy of the partition-coefficient in the case of nitrosophenol is of interest in view of the colloidal nature of the lipin phase.

Evidently the solubility of the substance in the lecithin is sufficiently high to obscure the surface-factor characteristic of colloidal systems. The results are analogous to those obtained in the case of the system water-phenol-protein [Cooper, *et al.*, 1912, 1923, 1927].

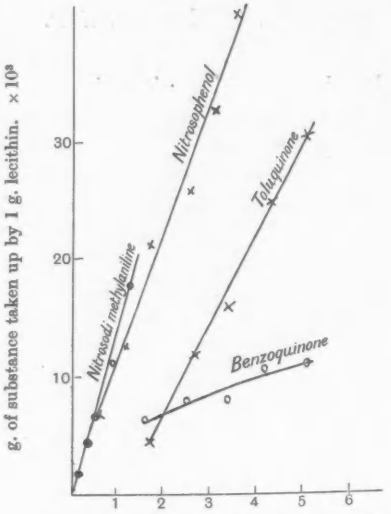
g. of substance taken up by 1 g. lecithin. × 10³
30
20
10
Nitrosophenol
Toluquinone
Nitrosodimethylaniline
Benzoquinone
1
2
3
4
5
6
g. of substance taken up by 1 cc. water. × 10³

Fig. 1.

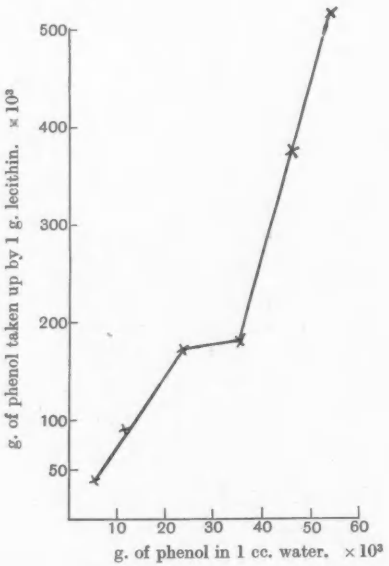
g. of phenol taken up by 1 g. lecithin. × 10³
500
400
300
200
100
50
10
20
30
40
50
60
g. of phenol in 1 cc. water. × 10³

Fig. 2.

The nitroso-compounds and quinones had no precipitating action on the lecithin, but with phenol precipitation of the lipin as a viscous mass occurred at a phenol concentration of 1–2 %, and it is at this point that a change in the distribution-ratio took place, as in the case of proteins (Fig. 2).

The results with *p*-nitrosodimethylaniline and toluquinone suggest that these compounds undergo molecular association when dissolved in lecithin, and in Table V the distribution-ratios obtained by dividing the square-root of the concentration in the lipin phase are tabulated.

Table V.

	Concentration in		
Water phase (*A*) (g. per litre)	Lipin phase (*B*) (g. per 1000 g.)	Square-root of $B = C$	Distribution-ratio C/A
Toluquinone			
5·136	30·60	5·532	1·07
4·303	24·87	4·990	1·16
3·440	15·92	3·987	1·16
2·720	11·94	3·450	1·26
1·684	4·48	2·012	1·19
p-Nitrosodimethylaniline			
1·301	17·78	4·219	3·24
0·952	11·19	3·347	3·51
0·796	9·01	3·000	3·89
0·583	6·59	2·567	4·40
0·398	4·28	2·069	5·20
0·194	1·65	1·285	6·63

In the case of toluquinone, the new distribution-ratio is almost constant over a wide range of concentration, showing that toluquinone is bimolecular when dissolved in lecithin. On the other hand, this constancy is not observed with *p*-nitrosodimethylaniline, the distribution-ratio being abnormally large at low concentration. It is probable that the nitroso-compound undergoes molecular association in lecithin, but is at the same time adsorbed to a certain extent, thus causing the distribution-ratio to vary.

Summary.

1. An essential condition for the bactericidal action of the nitrosoanilines is the maintenance of the amino-nitrogen in the tervalent state. The hydrochloride and methiodide are weak germicides.

2. The nitroso-compounds are slowly-acting disinfectants, exerting only a small bactericidal action in short periods, but this increases greatly in intensity in periods of 24–48 hours.

3. The nitroso-compounds have little or no action on amino-acids and proteins, but react gradually with nucleic acid forming a dark green insoluble product.

It is concluded therefore that the nitroso-compounds owe their slow germicidal action and marked inhibitory power to their gradual chemical interaction with the nuclear constituents of the cell, thus interfering with and retarding the biochemical mechanism of growth.

The authors desire to express their best thanks to the Department of Scientific and Industrial Research for a maintenance grant which has made it possible to carry out this investigation.

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III. STUDIES ON THE RELATIONSHIP BETWEEN CHEMICAL CONSTITUTION AND PHYSIOLOGICAL ACTION.

PART II. THE MIOTIC ACTIVITY OF URETHANES DERIVED FROM THE ISOMERIC HYDROXY-BENZYLDIMETHYLAMINES.

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(*Received November 27th, 1928.*)

In Part I of this series, the author [1926] synthesised, and tested for miotic activity, a number of substituted phenyl esters of substituted carbamic acids, thereby demonstrating that miotic activity was frequently associated with urethanes of this type. While certain variations could be made in the urethane grouping without necessarily destroying the activity of the compounds, greatest activity was observed with those urethanes which were esters of methylcarbamic acid. It was further found that both the nature and position of the basic group, which was present in all the compounds examined, could be varied without in all cases destroying the physiological properties of the urethane grouping. Nevertheless, an examination of the isomeric dimethylaminophenyl esters of methyl- and ethyl-carbamic acids showed that the power of these compounds to produce a contraction of the pupil varied according to the relative positions of the basic and urethane groupings, and, further, that conversion of the tertiary basic group into a quaternary ammonium grouping exerted a great influence on this power, in some cases increasing, and in others diminishing, the miotic activity. By application of the polarity theory the deduction was made that, for each series of isomeric urethanes examined, greatest activity was exhibited by those urethanes derived from the phenols with the smallest acidic properties, and the same conclusion can be reached by application of the more modern electronic theories.

In view of these results it appeared of interest to examine a new series of isomeric urethanes. The urethanes derived from the isomeric hydroxybenzyldimethylamines have therefore been prepared and tested for miotic activity. This particular series was chosen for two reasons. Firstly, it was desired to obtain substances the salts of which would not undergo appreciable hydrolytic dissociation in solution, as was the case when the basic group was attached directly to the benzene ring, since these would be more suitable for complete

pharmacological investigation. Secondly, it was hoped to gain further information regarding the relation between the miotic activity of the urethane and the acidic strength of the phenol from which it was derived.

The methods employed for the preparation of these compounds are described below and only one point requires comment. Whereas the dimethylaminophenols react smoothly with methyl *iso*cyanate to give the urethanes described in Part I, the reaction in the case of the hydroxybenzyldimethylamines is much more complicated. Using the conditions previously employed, the products from the *ortho*- and *para*-compounds appear to consist of a mixture of substances from which the required urethane can only be isolated with difficulty, if at all. By suitably modifying the conditions these difficulties have been overcome and, by strictly adhering to the conditions described in the experimental portion, the *N*-methylurethanes have been obtained in practically quantitative yield. In the case of the *N*-ethylurethanes, however, the difficulties have not been completely surmounted and these have only been isolated in the form of their salts.

Of the urethanes of this series which it has been possible to test, miotic activity has been confined to those which are esters of methylcarbamic acid; no activity has been observed with *N*-ethyl- or *N*-phenyl-urethanes. This corresponds with the results described in Part I, from which it was concluded that greatest activity was associated with esters of methylcarbamic acid. In conformity with the previous results, it has further been found that the activities of the three isomeric *N*-methylurethanes vary in intensity, the estimated order of the activities of the hydrochlorides being *ortho-* > *para-* > *meta-*. Not only does this order correspond with that of the lower homologues, but the difference between the activities of the *ortho-* and *para*-compounds has again proved to be much greater than that between the *para-* and *meta-*. Conversion of the tertiary basic group into the quaternary ammonium group intensifies the activity of the *ortho-*, diminishes that of the *meta-*, and abolishes that of the *para*-compound. This accords with the behaviour of the urethanes from the dimethylaminophenols in so far as the activities of the salts of the tertiary bases are different from those of the quaternary salts, but not as regards the direction in which the change occurs with the different isomerides. The author had hoped that it would have been possible to deduce the relative acidic strengths of the phenolic groups in the isomeric hydroxybenzyldimethylamines (and in their quaternary ammonium salts) from electronic theories, but unfortunately it appears impossible to do so in the present state of the development of these theories. Hence, while it is not improbable that the urethanes of these series which are the most active miotics are again those derived from the least acidic phenols, a discussion of this point must be deferred until it has been possible to measure directly the acidic strengths in question.

In Part I it was tacitly assumed that the miotic activity of the active urethanes was associated with the fact that they were phenyl, as distinguished

from alkyl, esters of carbamic acids, but no proof was advanced that similar activity might not be exhibited by urethanes derived from alcohols, provided these contained a basic group. The methylurethanes of two amino-alcohols, namely, dimethylaminoethyl alcohol and tropine, have accordingly been prepared. As anticipated, neither possessed miotic properties. Owing to the failure to obtain the hydrochloride of the first-named urethane in a crystalline condition the substance was tested in the form of its methiodide, which was therefore the methylurethane of choline iodide. It is interesting to note that the methylurethane of tropine exhibited neither miotic nor mydriatic properties. That it is not a mydriatic corresponds, of course, with the fact that the tropine is esterified with an aliphatic acid.

Experimental.

Urethanes derived from m-*hydroxybenzyldimethylamine.*

Methylurethane of m-*hydroxybenzyldimethylamine.* Finely powdered *m*-hydroxybenzyldimethylamine (1 g.), prepared according to the author's method [1927], was treated with excess of methyl *iso*cyanate. The phenol dissolved slowly with slight evolution of heat. The mixture was cooled in ice and, after 24 hours, light petroleum added. This caused the precipitation of only a small amount of oil. Hence all solvents were removed by distillation under diminished pressure, when the oily residue slowly crystallised. It was recrystallised by addition of light petroleum to its solution in benzene, when it formed thick plates which melted at 86°.

The *hydrochloride* separated from a solution of the base in alcoholic hydrochloric acid. Recrystallised from alcohol, it formed radiating clusters of prisms which melted at 175° with effervescence.

Analysis. 0·2448 g. gave 0·1423 g. AgCl.
9·06 mg. contained 1·05 mg. N (micro-Kjeldahl).
Cl found, 14·4 %; calc., 14·5 %.
N found, 11·6 %; calc., 11·5 %.

The *methiodide* was prepared by treating the base with excess methyl iodide and sufficient methyl alcohol to cause the solid to dissolve. After standing overnight the methiodide was precipitated by addition of dry ether. When recrystallised from alcohol it formed aggregates which melted at 162°.

Ethylurethane of m-*hydroxybenzyldimethylamine.* The finely powdered phenol was dissolved in ethyl *iso*cyanate and allowed to stand for 24 hours. Evaporation of the excess of ethyl *iso*cyanate under diminished pressure left an oil which could not be induced to crystallise. It was therefore divided into two portions, one of which was dissolved in alcoholic hydrochloric acid. After a short time the *hydrochloride* separated. It was recrystallised from alcohol when it formed rectangular tablets which melted at 156°.

Analysis. 0·2033 g. gave 0·1140 g. AgCl.
Cl found, 13·9 %; calc., 13·7 %.

The second portion of the crude base was dissolved in acetone and the solution treated with methyl iodide. The *methiodide* separated on standing. When recrystallised from alcohol it formed clusters of irregular plates which melted at 155–156° with effervescence.

Analysis. 0·1452 g. gave 0·0926 g. AgI.
I found, 34·5 %; calc., 34·9 %.

The results of a number of experiments made under varying conditions were practically identical with that of the above example. In no case was the base obtained in a solid condition, although the yield of salt was practically theoretical. Thus, in one experiment, 0·5 g. of *m*-hydroxybenzyldimethylamine yielded 1·1 g. of the methiodide of the ethylurethane.

Phenylurethane of m-*hydroxybenzyldimethylamine.* One molecular proportion (0·36 cc.) of phenyl *iso*cyanate was added to a cooled solution of 0·5 g. of the phenol in 5 cc. of benzene and the mixture left overnight in the refrigerator. The solution was then filtered to remove a small quantity of non-basic material which had separated. Evaporation of the solvents under diminished pressure yielded an oil which rapidly crystallised. When recrystallised from benzene, in which it is fairly soluble, it formed prisms which melted at 93°.

The *hydrochloride* separated when the base was dissolved in alcoholic hydrochloric acid. Recrystallised from alcohol it formed short prisms which melted at 175°.

Analysis. 0·2580 g. gave 0·1185 g. AgCl.
9·88 mg. contained 0·89 mg. N (micro-Kjeldahl).
Cl found, 11·4 %; calc., 11·6 %.
N found, 9·0 %; calc., 9·1 %.

Urethanes derived from p-*hydroxybenzyldimethylamine.*

Methylurethane of p-*hydroxybenzyldimethylamine.* One molecular proportion (0·24 cc.) of methyl *iso*cyanate was added to a cooled solution of 0·5 g. of the phenol in 5 cc. of benzene, causing the immediate formation of a slight precipitate. After standing overnight, light petroleum was added and a small amount of hygroscopic precipitate removed by filtration. The solvents were then evaporated at low temperature, when the residual oil crystallised. It was recrystallised by addition of light petroleum to a concentrated solution in benzene, when it formed a felted mass of fine crystals which melted at about 72°.

The *hydrochloride* separated when the base was dissolved in alcoholic hydrochloric acid. When recrystallised from alcohol it formed prisms with pyramid ends which melted at about 180° with effervescence.

Analysis. 0·2455 g. gave 0·1423 g. AgCl.
Cl found, 14·3 %; calc., 14·5 %.

The *methiodide* was prepared by addition of methyl iodide to a solution of the urethane in acetone. When recrystallised from alcohol it formed lustrous plates which melted at 188° with decomposition.

The *methochloride* was prepared by shaking for one hour a solution of the methiodide in methyl alcohol with a suspension of freshly precipitated silver chloride in methyl alcohol. The suspension was then filtered and the filtrate evaporated, when the residue, which consisted of the methochloride, crystallised. It was recrystallised from alcohol by the addition of dry ether, when it formed clusters of radiating needles which melted at about 110° with effervescence.

Analysis. 0·1575 g. gave 0·0855 g. AgCl.
Cl found, 13·4 %; calc., 13·7 %.

Ethylurethane of p-*hydroxybenzyldimethylamine.* The phenol (1 g.) was dissolved in dry acetone (10 cc.), and the cooled solution treated with one molecular proportion (0·6 cc.) of ethyl *iso*cyanate. The solvents were evaporated on the following day, when the oily residue partly crystallised. It was found impossible, however, to separate the crystals. The product was therefore dissolved in alcoholic hydrochloric acid. On addition of dry ether to this solution the *hydrochloride* separated as an oil which, after repeatedly washing with dry ether, mixtures of ether and acetone, and mixtures of ether and alcohol, finally crystallised. For complete purification it was necessary to recrystallise it three times by addition of ether to its alcoholic solution, when it formed rosettes of plates which melted at 165° with effervescence.

Analysis. 0·2105 g. gave 0·1148 g. AgCl.
Cl found, 13·5 %; calc., 13·7 %.

The same hydrochloride was obtained from the product of the action of an excess of ethyl *iso*cyanate on the phenol.

The *methiodide* was prepared by the addition of methyl iodide to a solution of the crude urethane in methyl alcohol, from which it was precipitated by the addition of dry ether. When recrystallised from alcohol it formed irregular plates which melted at 186°.

Analysis. 0·1787 g. gave 0·1170 g. AgI.
I found, 35·4 %; calc., 34·9 %.

Phenylurethane of p-*hydroxybenzyldimethylamine.* One molecular proportion (0·36 cc.) of phenyl *iso*cyanate was added to a cooled solution of 0·5 g. of the phenol in 5 cc. of benzene. The urethane quickly separated as an amorphous precipitate. This was filtered and crystallised from alcohol, when it formed thick prisms which melted at 126°.

The *hydrochloride* crystallised from alcohol in rosettes of needles which melted at 182°.

Analysis. 0·2455 g. gave 0·1132 g. AgCl.
12·72 and 14·19 mg. contained respectively 1·13 and 1·30 mg. N (micro-Kjeldahl).
Cl found, 11·4 %; calc., 11·6 %.
N found, 8·9 and 9·1 %; calc., 9·1 %.

Urethanes derived from o-*hydroxybenzyldimethylamine.*

Methylurethane of o-*hydroxybenzyldimethylamine.* The phenol (0·5 g.) was dissolved in 5 cc. of ether and a trace of metallic sodium added. This dissolved with the evolution of hydrogen. The solution was then cooled and one

molecular proportion (0·24 cc.) of methyl *iso*cyanate added. After some hours, a small quantity of flocculent material which had separated was removed by filtration and the filtrate evaporated at low temperature, when the residue crystallised. When recrystallised by addition of light petroleum to its solution in benzene it formed flat prisms which melted at 76°.

The *hydrochloride* was hygroscopic and could not be obtained crystalline.

The *methiodide* slowly separated from a solution of the base in acetone to which an excess of methyl iodide had been added. When recrystallised from alcohol it formed small prisms which melted at 175° with decomposition.

Analysis. 0·1565 g. gave 0·1045 g. AgI.
I found, 36·1 %; calc., 36·3 %.

Ethylurethane of o-hydroxybenzyldimethylamine. A number of experiments were carried out under different conditions, but in no case was it possible to isolate the urethane in a pure state. In the following experiment, however, a small quantity was obtained in the form of its methiodide. Attempts which have since been made to prepare larger quantities of this methiodide have unfortunately not been successful, although the experiments have been carried out under conditions as nearly identical as possible.

The phenol (0·5 g.) was dissolved in 5 cc. of dry ether and a trace of metallic sodium added. This dissolved with the evolution of hydrogen, when one molecular proportion (0·3 cc.) of ethyl *iso*cyanate was added. After standing overnight the solution was filtered from a small quantity of insoluble material and the filtrate evaporated. An oil was thus obtained which showed no signs of crystallising. It was therefore dissolved in acetone and excess of methyl iodide added. Addition of ether then precipitated an oily methiodide which, after standing till the next day, crystallised. It was filtered and the sticky substance thus obtained recrystallised from acetone, when it formed prisms which melted at 149° with effervescence.

Analysis. 16·16 and 14·49 mg. contained respectively 1·21 and 1·14 mg. N (micro-Kjeldahl).
N found, 7·5 and 7·8 %; calc., 7·7 %.

Phenylurethane of o-hydroxybenzyldimethylamine. A solution of 0·5 g. of the phenol in 5 cc. of benzene was treated with one molecular proportion (0·36 cc.) of phenyl *iso*cyanate. Some crystalline material separated slowly. This was filtered and washed with light petroleum (yield 0·48 g.). It was then warmed with a small volume of benzene, filtered from some insoluble crystals of a non-basic substance, concentrated, and placed in the refrigerator. A small quantity of the required urethane separated in the form of plates which melted at about 90°.

Analysis. 11·89 mg. contained 1·23 mg. N (micro-Kjeldahl).
N found, 10·4 %; calc., 10·4 %.

This urethane is apparently so unstable that considerable losses occur during recrystallisation.

The *methiodide* was prepared by warming the urethane with methyl iodide, dissolving the oil so produced in methyl alcohol, and then adding acetone and

dry ether, when the methiodide separated as a white solid. It was recrystallised by the slow addition of dry ether to its solution in acetone containing the minimum amount of methyl alcohol, when it formed rosettes of flat prisms which melted at 171°.

Urethanes derived from amino-alcohols.

Methylurethane of choline iodide. 1 g. of hydroxyethyldimethylamine, cooled in a freezing mixture, was treated with excess of methyl *iso*cyanate. After standing overnight, benzene and light petroleum were added, the mixture stirred, and the supernatant liquid decanted from a small amount of insoluble oil which was not further examined. The solvents were then evaporated, when a colourless liquid was obtained which would neither crystallise nor yield a solid hydrochloride. It was therefore dissolved in methyl iodide. A somewhat vigorous reaction took place and the methiodide separated in crystalline form. After addition of acetone, the crystals were filtered off and recrystallised from alcohol when they formed lustrous plates which melted at 174°.

Analysis. 0·1144 g. gave 0·0923 g. AgI.
I found, 43·6 %; calc., 44·1 %.

Methylurethane of tropine. The tropine, prepared from atropine, was dissolved in methyl *iso*cyanate and allowed to stand for some hours. The excess of methyl *iso*cyanate was then removed under diminished pressure and the residue crystallised from benzene, in which it is fairly soluble, when it formed rectangular plates which melted at about 126°.

The *hydrochloride* was precipitated as a sticky solid by addition of ether to a solution of the base in alcoholic hydrochloric acid. It was crystallised from a mixture of dry ether and alcohol, when it melted at 263° with decomposition.

Miotic activity of urethanes.

The tests for miotic activity were carried out on cats as described in Part I. In general, 2 % solutions of the urethane in Ringer's solution were employed. In the case of the methylurethane of *o*-hydroxybenzyldimethylamine, the hydrochloride of which has not been obtained in crystalline form, the base was dissolved in the calculated quantity of hydrochloric acid. The results obtained are given in the following table in which the estimated relative intensities of the activities of the active urethanes are indicated.

Substance	Miotic activity
m-NH $(CH_3).CO.O.C_6H_4.CH_2.N(CH_3)_2$, HCl	+ +
m-NH $(CH_3).CO.O.C_6H_4.CH_2.N(CH_3)_3I$	+
m-NH $(C_2H_5).CO.O.C_6H_4.CH_2.N(CH_3)_2$, HCl	−
m-NH $(C_2H_5).CO.O.C_6H_4.CH_2.N(CH_3)_3I$	−
m-NH $(C_6H_5).CO.O.C_6H_4.CH_2.N(CH_3)_2$, HCl	−
p-NH $(CH_3).CO.O.C_6H_4.CH_2.N(CH_3)_2$, HCl	+ + +
p-NH $(CH_3).CO.O.C_6H_4.CH_2.N(CH_3)_3I$	−
p-NH $(C_2H_5).CO.O.C_6H_4.CH_2.N(CH_3)_2$, HCl	−
o-NH $(CH_3).CO.O.C_6H_4.CH_2.N(CH_3)_2$, HCl	+ + + +
o-NH $(CH_3).CO.O.C_6H_4.CH_2.N(CH_3)_3I$	+ + + + +
o-NH $(C_2H_5).CO.O.C_6H_4.CH_2.N(CH_3)_3I$	−
NH $(CH_3).CO.O.CH_2.CH_2.N(CH_3)_3I$	−

SUMMARY.

1. The methyl-, ethyl- and phenyl-urethanes of the isomeric hydroxybenzyldimethylamines, and the methylurethanes of choline iodide and tropine have been prepared and, where possible, tested for miotic activity.

2. Miotic activity was confined to the methylurethanes of the isomeric hydroxybenzyldimethylamines, the urethanes of choline iodide and tropine being inactive in 2 % solutions. This confirms the view that activity is associated with the fact that the miotics are substituted phenyl, as distinguished from alkyl, esters of carbamic acids.

3. The activities of the methylurethanes of the isomeric hydroxybenzyldimethylamines are estimated to be in the order *ortho-* > *para-* > *meta-*. Conversion of the tertiary basic group into the quaternary ammonium group increased the activity of the *ortho*-compound, diminished that of the *meta-*, and abolished that of the *para-*.

The expenses of this investigation, which has been carried out during the tenure of a Carnegie Teaching Fellowship, has been met by grants from the Earl of Moray Research Fund of this University.

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IV. THE DENATURATION OF PROTEINS. PART V.

DENATURATION BY ACID.

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In earlier work carried out in this laboratory by P. S. Lewis [1926, 1927] on the heat denaturation of oxyhaemoglobin and egg-albumin it was shown that the unimolecular velocity constant characterising the process had a minimum value at the neutrality point of water (namely $p_H = 6{\cdot}76$ at 37°), and, further, that the process appeared to be catalysed approximately to the same extent by hydrogen ions and by hydroxyl ions. In the region of minimal velocity it was also found that the denaturation possesses an extremely high critical increment, namely of the order 77,000 cals. for oxyhaemoglobin, and 130,000 cals. for albumin. The temperature range for which these values were obtained was in round numbers 60° to 70°.

In view of these facts it was considered to be of importance to extend the p_H range as far as possible in the direction of increasing acidity, such extension necessarily involving the employment of lower ranges of temperature in order to deal with reaction velocities which would not be immeasurably rapid.

The present communication contains a summary of the results obtained. The proteins investigated, namely oxyhaemoglobin and albumin, were prepared and purified by the same methods as those employed by Lewis [1926, 1927]. Lewis's methods of determining the extent of denaturation at the various time intervals were also followed, with the exception that for denaturation at relatively low temperatures, such as 18°, it was necessary not only to cool the sample withdrawn, but also to adjust its p_H by addition of alkali to that of the isoelectric point to ensure that the denaturation itself is stopped, and also that all the protein denatured is precipitated.

The velocity of denaturation has been determined at 18°, 25°, 37° and 45°, over different ranges of p_H. The velocity constants given in the tables are in all cases calculated from the ordinary unimolecular expression in which the logarithm is to the base e and time is expressed in seconds. The stock solutions contained 0·5 % ammonium sulphate. It is known that this concentration of the salt does not appreciably affect the velocity of denaturation, and at the same time the ammonium sulphate not only acts as a preservative, but also ensures that the flocculation of the denatured protein shall be very rapid compared with the denaturation process itself.

All the results quoted are the mean values of concordant experiments. For the case of oxyhaemoglobin the average divergence of the mean values does not exceed 2 to 3 %; for albumin the error is somewhat larger. As a rule, the denaturation was followed to the extent of over 50 % in any individual run. The p_H was determined by means of the quinhydrone electrode at 25° in all cases.

Acid denaturation of oxyhaemoglobin.

Temperature 18°		Temperature 25°	
p_H of solution	$k_{uni} \times 10^4$	p_H of solution	$k_{uni} \times 10^4$
4·08	10·95	4·08	17·61
4·13	8·19	4·13	12·49
4·30	4·58	4·32	5·25
4·39	2·76	4·39	4·30
4·40	2·71	4·41	3·73
4·49	1·69	4·52	2·29
4·56	1·07	4·64	1·27
—	—	4·77	0·51

Temperature 37°		Temperature 45°	
p_H of solution	$k_{uni} \times 10^4$	p_H of solution	$k_{uni} \times 10^4$
4·11	30·65	—	—
4·15	22·71	4·23	25·30
4·31	12·26	4·45	11·10
4·48	6·31	4·52	7·26
4·51	5·15	4·63	4·36
4·60	3·22	4·74	2·74
4·68	2·19	4·90	1·21
4·85	0·80	5·10	0·59
		5·50	0·096

Acid denaturation of egg-albumin.

In connection with this protein previous workers have observed a change in p_H during the course of heat denaturation. Consequently in the present series of measurements several determinations of p_H values were made by means of the quinhydrone electrode at various points during the course of a run, and it can be quite definitely stated that, over the temperature range and p_H range investigated by the writer, there appeared to be no sensible alteration in p_H as a consequence of denaturation.

Temperature 25°		Temperature 37°	
p_H of solution	$k_{uni} \times 10^5$	p_H of solution	$k_{uni} \times 10^5$
1·02	1·59	1·02	30·0
1·17	1·35	1·32	8·83
1·35	0·82	1·46	7·00
1·52	0·64	1·58	5·56
1·72	0·48	1·62	5·68
		1·80	3·57
		1·99	2·16
		2·25	1·08
		2·55	0·91
		3·83	0·06

Temperature 45°

p_H of solution	$k_{uni} \times 10^4$	p_H of solution	$k_{uni} \times 10^4$
1·34	3·88	2·18	0·96
1·50	2·84	2·30	0·82
1·66	2·45	2·39	0·65
1·96	1·61	2·68	0·35
1·98	1·73	3·52	0·12

The critical increment.

This quantity, E, which represents the energy of activation of the denaturation process is calculated from the usual expression

$$\log \frac{k_2}{k_1} = \frac{E}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right)$$

where k_2 and k_1 are the observed velocity constants at the two temperatures T_2 and T_1. The values of E corresponding to the temperature range and to a number of p_H values within the limits quoted above are recorded in the following tables. It may be pointed out that errors in the individual velocity constants become magnified in the calculation of the E values therefrom. The values quoted are consequently rounded.

Critical increment of the acid denaturation of oxyhaemoglobin.

p_H	Temp. range 18°–25° E in cals.	Temp. range 25°–37° E in cals.	Temp. range 37°–45° E in cals.
4·1	11,900	—	—
4·2	8,600	11,100	—
4·3	8,300	14,100	(6,600)
4·4	9,100	12,800	(4,000)
4·5	11,300	11,700	12,000
4·6	—	9,100	12,000
4·7	—	12,400	13,900
4·8	—	—	12,500

Critical increment of the acid denaturation of egg-albumin.

p_H	Temp. range 25°–37° E in cals.	p_H	Temp. range 37°–45° E in cals.
1·1	36,200	1·4	36,200
1·2	35,900	1·5	37,000
1·3	35,800	1·6	39,000
1·4	35,300	1·7	40,000
1·5	35,400	1·8	40,000
1·6	36,400	1·9	43,100
1·7	36,800	2·0	45,300
		2·1	48,100
		2·2	49,000
		2·3	48,200

Discussion.

Even when full allowance is made for inevitable experimental error the striking result, shown in the foregoing tables, is that in the region of moderate acidity the critical increment of denaturation, both for oxyhaemoglobin and for egg-albumin, is very much lower than it is in the neighbourhood of the

neutrality point of water in which region the process is known as heat denaturation. It is not proposed in the present paper to attempt to offer any explanation of this. It is not even known with certainty, as yet, whether or not the moderate values of E which hold for the low p_H range and relatively low temperature tend to merge gradually into the extremely high values of E obtained at the high p_H range and relatively high temperature characteristic of heat denaturation. If the change is a gradual one this would constitute an argument of some weight pointing to the fundamental identity of acid denaturation and heat denaturation respectively. Further work is being undertaken in this laboratory in this connection.

As we have just had occasion to raise the question of the relation of heat denaturation to denaturation by acid, it may not be out of place to draw attention to yet another mode whereby denaturation is said to be brought about, namely, mechanical denaturation. In this connection certain results obtained by Wu and Ling [1927] may be cited to the effect that, in contrast to other forms of coagulation which involve denaturation as a distinct stage, mechanical treatment (shaking) leads directly to the observation that maximum velocity occurs at the isoelectric point. Under ordinary conditions the speed of denaturation is slow compared with that of the (subsequent) flocculation of denatured material. As a consequence of mechanical treatment it would seem that the denaturation process has been greatly speeded up, so that the total observed effect is essentially a coagulation rate which would naturally be a maximum at the isoelectric point. So far as this evidence goes it affords some slight support to the view that denaturation is essentially a distortion or opening up of the protein unit, usually brought about as a consequence of localised chemical changes in the unit, but possibly much more rapidly brought about by mechanical treatment.

The influence of formaldehyde upon the flocculation of denatured protein.

Flocculation of a protein is the process which is subsequent to denaturation and is rendered possible by it. Flocculation is usually ascribed to the interaction of amino- and carboxyl groups situated on contiguous colloid units as a consequence of which these units increase in size by aggregation and eventually are coagulated. If this view be correct, then, if by any means either of these groups be blocked or inactivated, the resulting protein should not exhibit flocculation. To this end formaldehyde at various concentrations was caused to react with protein and the resulting material examined at various p_H values including the isoelectric point itself.

According to Harris [1923] the simplest mode of reaction of formaldehyde with an amino-group will lead to the formation of a methylene-imino-linkage, such reaction, however, not going to completion but attaining an equilibrium.

The results obtained, probably owing to the existence of the equilibrium

just referred to, were not as conclusive as had been hoped. They may be very briefly summarised as follows.

(*a*) In solutions of denatured oxyhaemoglobin containing formaldehyde (up to 0·5 %), some precipitation occurs on the acid side of the isoelectric point as far as p_H 3, and at the isoelectric point. There is no precipitation at p_H 7 and at higher p_H values.

(*b*) In solutions of denatured oxyhaemoglobin not containing formaldehyde there is complete precipitation at the isoelectric point and within a certain range on either side thereof, *i.e.* precipitation is still practically complete even at p_H 8 and decreases as the solution becomes more alkaline, whilst on the acid side the precipitate is still fairly complete even as far as p_H 3. The range over which precipitation occurs is greater in the absence of formaldehyde than in its presence.

As in the case of denatured oxyhaemoglobin the p_H range and degree of precipitation of denatured egg-albumin are both decreased by the addition of formaldehyde, and the greater the amount of formaldehyde added the greater the decrease.

The behaviour of both proteins towards formaldehyde may therefore be taken as evidence in support of the view that flocculation involves the amino- (and consequently the carboxyl) groups.

Summary.

1. The unimolecular velocity constants of the denaturation, by means of acid, of the two proteins oxyhaemoglobin and egg-albumin, have been determined at various temperatures and over a considerable p_H range.

2. From the observed velocity constants the corresponding values of the critical increments of activation for different temperature ranges have been calculated. It is found that for the acid denaturation of oxyhaemoglobin the critical increment has an average value of 12,000 cals. For egg-albumin the corresponding quantity lies between 36,000 and 48,000 cals.

3. Attention is drawn to the marked difference in the magnitude of the critical increments recorded and those already obtained for so-called heat denaturation, namely, 77,000 cals. for oxyhaemoglobin and 130,000 cals. for egg-albumin. Whether the values for acid denaturation merge into those for heat denaturation as the acidity and temperature range are altered to those usually associated with heat denaturation is under investigation.

4. The influence of formaldehyde upon the flocculation of denatured protein has been examined. It is found that the p_H range over which precipitation occurs is greater in the absence of formaldehyde than in its presence. This serves to confirm the generally accepted view that flocculation involves the amino- (and consequently the carboxyl) groups.

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V. THE DETERMINATION OF SMALL QUANTITIES OF STARCH IN VEGETABLE TISSUE.

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In some work on the reserve foods of the bracken fern, it was found necessary to use an accurate method of estimating starch in small quantities. Methods commonly in use which involve comparison of determinations of the sugar mixture, obtained after hydrolysis, by reduction of copper salts, and by the polarimeter, were not suitable as the quantities were too small for the use of the polarimeter. A method was therefore devised by which the copper-reducing power of the hydrolysed starch in the plant material was related to the reducing power of known quantities of a carefully purified starch preparation. Such a method does not give absolute values as the reducing power of starches of different origins is not the same. It does, however, give values comparable with each other for any one material and this is sufficient for many purposes, such as the comparison of the amounts of starch present at different stages of development.

The standard starch used for comparisons was prepared as follows. Lintner's soluble starch was washed carefully by decantation with six changes of distilled water. The remaining water was removed by washing in a similar way with three changes of absolute alcohol and drying to constant weight at 105°. Amounts of this preparation ranging from 0·1 to 0·8 g. were then weighed into clean dry conical flasks of 125 cc. capacity and 40 cc. of distilled water added. The mixture was then boiled for 30 seconds to emulsify the starch and cooled to room temperature. 5 cc. of a 0·2 % or 0·4 % aqueous solution of takadiastase (Parke Davis and Co., Ltd.) were added together with 0·05 cc. 5 % acetic acid and 0·2 cc. toluene, and the mixture was incubated at 36·8° for 24- or 48-hour periods. The same concentration of enzyme was used throughout; 0·2 % or 0·4 % gave very nearly the same results but smaller concentrations led to variations. Trials showed that after 48 hours the reaction was not quite complete, though even after 24 hours it was sufficiently slow to give consistent results, while shorter periods gave variable results.

The most suitable temperature was found by experiment to be 37°. At lower temperatures hydrolysis was slower and the copper-reducing power was not consistent. The temperature of the incubator was regulated to $\pm$ 0·3° about a mean of 36·8°, whilst that of the hydrolysis liquid varied $\pm$ 0·1° about the same mean. The addition of the acetic acid fixed the p_H at 5·5, at which value it remained constant throughout the reaction.

The copper reducing power was determined by Shaffer and Hartmann's method [1921] after several others, such as those of Pavy, Bertrand and Benedict, had been tried. All determinations were carried out in the following manner. The hydrolysis mixture, after the proteins, tannins and mucilages had been precipitated (see later), was neutralised to litmus and made up to a definite volume. 50 cc. of this solution were then pipetted into a conical 750 cc. flask and 50 cc. of Shaffer and Hartmann's reagent added, together with some pieces of unglazed plate. The mixture was brought to boiling point in four minutes, boiled for exactly five minutes, and cooled for three minutes under running water. As a constant source of heat an electric hot plate or a Bunsen burner, shielded from air currents by enclosure in a suitably ventilated tin, was used. A reflux condenser prevented concentration of the liquid by evaporation. The "cuprous titration" [Shaffer and Hartmann, 1921] was used exclusively.

A series of determinations upon different quantities of prepared starch was thus made. For use, it is convenient to plot the copper values (mg. copper reduced) against the weight of starch employed. The points fall very accurately in a straight line and from this graph, the amount of starch in the sample of plant material corresponding to the amount of copper reduced may be readily obtained. As has been said, this is only relative.

Table I. *Standard starch/copper values after hydrolysis with* 0·2 % *takadiastase.*

24 hours' incubation		48 hours' incubation	
Wt. of starch (mg.)	Wt. of copper reduced (mg.)	Wt. of starch (mg.)	Wt. of copper reduced (mg.)
31·0	47·4	20·0	36·1
40·0	56·2	30·0	50·0
53·2	67·4	41·0	62·4
60·0	76·8	59·8	82·4
71·4	86·5	69·7	92·9
89·9	106·9	78·5	105·6
100·1	116·5	80·0	108·4
110·0	123·0	88·1	115·9
119·9	135·3	103·5	129·7
140·0	157·9	108·7	134·7
—	—	119·9	152·4
—	—	139·9	171·0

The plant material, in this case bracken rhizome, was washed in water, killed by immersion in a bath of boiling methylated spirit to which sufficient ammonia (sp. gr. 0·8) had been added to make the total concentration of ammonia 1 %, and dried for 24 hours at 105°. The weight was then constant, as far as constant weight is possible with hygroscopic material. The dried material was powdered and could then be stored in well-stoppered bottles without showing any fungus growth even after 3 years.

A weighed amount of the dried tissue powder was placed in a tared Soxhlet extraction thimble and extracted for 24 hours with absolute alcohol in a Soxhlet apparatus, dried for 12 hours at 105° and weighed again. It was

absolutely necessary to conduct these weighings in a stoppered weighing-bottle. This extraction removed the sugars, chlorophyll and some of the proteins, and also rendered the material more easily soakable with water afterwards. 4 to 10 g. of the dry powder were weighed into a dry conical 125 cc. flask, emulsified with 40 cc. of water and hydrolysed in exactly the same manner as the standard starch. The reaction was stopped at the end of 24 or 48 hours by the addition of the reagent used for precipitating the remaining proteins, tannins, and mucilages.

Two such reagents were used with equal success and equally without effect upon the copper-reducing power of the products of hydrolysis.

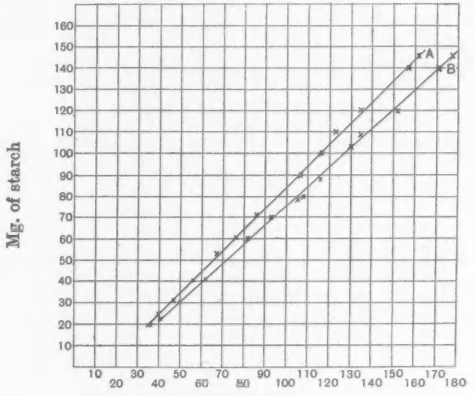

Fig. 1.

Standard starch/copper values

A. 24-hour period incubation.
B. 48-hour period incubation.

(1) *Lead acetate.* 20 cc. of a saturated solution of neutral lead acetate (A.R.) were added and the lead was almost entirely precipitated by the addition of 20 % potassium hydroxide, excess of the latter being avoided to prevent re-solution of any protein material. Upon filtration, a practically colourless liquid was obtained which contained no tannin, mucilage or protein. The excess of lead was removed by passing hydrogen sulphide, filtering, and subsequently aerating the filtrate for ten minutes to remove excess of hydrogen sulphide. The liquid was neutralised to litmus by potassium hydroxide and made up to a definite volume.

(2) *Mercury salts.* This was a modification of the method described by Thomas and Dutcher [1924], the mercury solution being prepared according to their formula. 20 cc. of this solution were added to the hydrolysis liquid after incubation. Solid sodium bicarbonate was then added in small quantities until frothing ceased, followed by 5 % sodium bicarbonate solution until

neutral to litmus. After filtration to remove the precipitate, which included the tannins, proteins and mucilages, the filtrate was acidified with dilute hydrochloric acid. The remaining mercury was precipitated by passing hydrogen sulphide and filtering. Excess of hydrogen sulphide was removed by aeration as before, and the liquid neutralised to litmus. The copper-reducing power was then determined in the same way as for the standard starch.

Successive estimations on the same sample of rhizome gave concordant results, *e.g.*

	Sample 1 (%)	Sample 2 (%)	Sample 3 (%)
	16·0	26·4	14·2
	16·2	26·0	14·4
	16·5	26·6	14·6
Average	16·2	26·3	14·4

A further test of the reliability of the method was given by estimating the amount of starch in a rhizome sample, adding a known weight of the prepared starch to another portion of the same material and making an estimation of the total starch.

The results of two such experiments show a very close agreement.

Wt. of starch in dried rhizome (g.)	Wt. of starch added (g.)	Wt. of starch determined (g.)
0·712	0·105	0·820
0·630	0·050	0·690

Summary.

This paper describes a method for comparative estimation of small amounts of starch in vegetable tissue.

I am indebted to Professor O. V. Darbishire, the late Mr C. Hunter, M.Sc., and to Dr Macgregor Skene, for many helpful suggestions.

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VI. NOTE ON THE CHARACTERISATION OF THE ANTHOCYANINS AND ANTHOCYANIDINS BY MEANS OF THEIR COLOUR REACTIONS IN ALKALINE SOLUTIONS.

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Fear and Nierenstein [1928] have pointed out that the colour reactions of the anthocyanins, which are indicators, should be examined in solutions of definite p_H and their contention is undoubtedly correct. It would be a mistake, however, to conclude that the earlier observations are entirely lacking in significance simply because this refinement has not hitherto been adopted, and, for example, the effect of elimination or modification of the hydroxyl in position 3 in the pyrylium ring on the colour reaction is very marked. Thus both luteolinidin chloride [Pratt and Robinson, 1925] and chrysanthemin chloride [Willstätter and Bolton, 1916] give violet solutions in aqueous sodium carbonate and these become blue on the addition of sodium hydroxide; cyanin chloride [Willstätter and Everest, 1913] gives a blue solution in aqueous sodium carbonate.

It was inferred that chrysanthemin chloride should be the 3-glucoside of cyanidin chloride and this has since been proved to be correct by the synthesis of the anthocyanin.

The examination of the reactions of the anthocyanidins in a range of buffered solutions has shown that this method is by far the most reliable for purposes of comparison and characterisation; it is of even greater value in this connection than a study of the absorption spectrum.

On the one hand various properties—*pseudo*-base formation and colour-base precipitation, ease of oxidation and so forth, are incidentally revealed and on the other, different anthocyanidins, as for instance, peonidin and malvidin, may exhibit identical absorption spectra [Schou, 1927].

A few examples have been selected to illustrate the method and it is hoped that the data will be found useful for the identification of anthocyanidins derived from natural sources.

Fear and Nierenstein state that cyanidin chloride from cyanin behaves differently from synthetic 3 : 5 : 7 : 3′ : 4′-pentahydroxyflavylium chloride and it is evident that by some misfortune they have not succeeded in obtaining a homogeneous specimen of the latter substance.

We repeat an emphatic affirmation that pure synthetic 3 : 5 : 7 : 3′ : 4′-pentahydroxyflavylium chloride, best prepared by hydrolysis of its benzoyl derivative, exhibits no divergences from cyanidin chloride and this is true, to the minutest detail, in regard to the tests described below.

Indeed the constitution of cyanidin chloride in its main structural outlines (carbon and oxygen skeleton) is established as firmly as that of any other compound and no attempt [Malkin and Nierenstein, 1928] to reconcile the results of Nierenstein [1921] in the field of catechin chemistry with the conflicting results of Freudenberg and Purrman [1924], by suggesting a modified cyanidin formula, can succeed. The explanation of the discrepancies must be sought elsewhere.

Experimental.

The buffered solutions were prepared from the tubes of "Universal Buffer Mixture," supplied by the British Drug Houses Limited. This mixture is stated to be compounded in accordance with a formula of Prideaux and Ward [1924] but the exact composition has not been disclosed.

It may happen that this excellent commercial product may be unobtainable in some quarters or at some future time and as it is in any case necessary to guarantee the reproducibility of our results we think it well to mention that a mixture of 0·04 g. mol. each of phenylacetic acid, boric acid and potassium dihydrogen phosphate appeared to be the equivalent of the contents of one of the tubes supplied. Phenylacetic acid, boric acid and potassium dihydrogen phosphate (0·02 g. mol. each), together with n cc. of 0·2 N sodium hydroxide, were dissolved in water and made up to 1000 cc. The solutions used and the approximate p_H of each, as found by means of indicators, were the following: (1) $n = 0$; p_H, 3·2; (2) $n = 25$; p_H, 3·8; (3) $n = 50$; p_H, 4·4; (4) $n = 75$; p_H, 5·0; (5) $n = 100$; p_H, 5·6; (6) $n = 125$; p_H, 6·2; (7) $n = 150$; p_H, 6·8; (8) $n = 175$; p_H, 7·4; (9) $n = 200$; p_H, 8·0; (10) $n = 225$; p_H, 8·6; (11) $n = 250$; p_H, 9·2; (12) $n = 275$; p_H, 9·8; (13) $n = 300$; p_H, 10·4; (14) $n = 325$; p_H, 11·0; (15) $n = 350$; (16) $n = 400$; (17) $n = 450$.

The anthocyanidin chloride (25·00 mg.) was dissolved in 98 % alcohol (100 cc.) and 1 cc. of the solution added from a burette to 10 cc. of the buffered solutions or of other solutions mentioned below.

Apigeninidin chloride. The alcoholic solution was orange; 1 % hydrochloric acid, bright canary yellow unchanged after 24 hours; 20 % hydrochloric acid, bright canary yellow and after 24 hours almost the whole of the salt had separated in clusters of microscopic prisms; (1) more orange yellow than the solution in 1 % hydrochloric acid, unchanged after 24 hours; (2) more intense yellowish orange, slight fading after 24 hours; (3) reddish orange fading slightly during 24 hours; (4) orange red, after 24 hours, long, slender crystals of colour base deposited; (5) darker orange red, after 24 hours colour base crystallised in slender needles; (6) brownish red, after 24 hours slight fading; (7) brownish red, a little bluer, same as (6) on keeping; (8) still a little

bluer brownish red, same as (6) on keeping; (9) bluish red, same as (6) on keeping; (10) cherry red, bluer than (9) and became like (6) on keeping; (11), (12), (13), (14), (15) same as (10); (16) same as (10) but became reddish orange after 24 hours; (17) same as (10) but became greenish yellow after 24 hours.

Pelargonidin chloride. 1 % and 20 % hydrochloric acid, orange red with bluish tinge in thin layers; (1) identical with hydrochloric acid solutions, colour faded but more slowly than in (2), (3) and (4), in 2 hours very faintly coloured, in 3½ hours colourless and remained so; (2) a little browner orange red, almost all colour disappeared in 17 minutes and colourless in 30 minutes; (3) bluish red fading quickly to pink and became colourless a little more rapidly than (2); (4) cherry red fading rapidly, still more so than (3); (5) bluish red, after 15 minutes rose coloured, after 3½ hours colourless (it should be noted that observations at all intermediate times could not be made and, for example, this solution may have been colourless after 2 hours); (6) violet red, after 3½ hours pale pink and after 24 hours still very weak red colour; (7) slightly bluer violet red, subsequent changes like (6); (8) like (7), after 3½ hours faded brownish pink, more intense than (7); (9) deeper violet, after 25 minutes reddish violet, after 24 hours weak reddish brown whereas (8) at this stage had a much weaker red colour; (10) violet, blue in thin layers, in 5 minutes pale violet, in 25 minutes reddish violet, after 24 hours weak reddish violet; (11) a little bluer than (10), after 25 minutes reddish violet, after 24 hours weak reddish violet; (12) a little bluer than (11) and dichroism less pronounced, after 10 minutes violet, after 25 minutes red violet, after 24 hours weak reddish violet; (13) again bluer, after 10 minutes violet, after 25 minutes red violet and after 24 hours very weak reddish brown; (14) similar, thin layers are greener blue, after 10 minutes violet, after 25 minutes violet, after 3½ hours weak reddish violet, after 24 hours very faint orange; (15), (16), (17) similar to (14) on mixing and after 24 hours, but became pale grey blue in 3 hours.

Cyanidin chlorides. 1 % and 20 % hydrochloric acid, eosin red, much bluer than pelargonidin; (1) red, slightly bluer than 1 % hydrochloric acid; the colour faded very slowly, after 18½ hours colourless; (2) bluish red, after 2½ minutes colour distinctly less intense, after 10 minutes considerable fading, after 45 minutes colourless; (3) reddish violet, after 10 minutes very weak, after 25 minutes colourless whereas at this stage both (2) and (4) retained weak colour; (4) bluer reddish violet, after 10 minutes colour much faded, after 45 minutes still weak violet, after 18½ hours colourless; (5) reddish violet, still bluer than (4), 15 seconds after mixing the colour-base separated giving opalescent solution; after 18½ hours precipitate of violet colour-base and colourless supernatant liquid; (6) bluer reddish violet of increased tinctorial intensity, no precipitate, after 18½ hours almost colourless—very faint pink; (7) violet tinged with red, after 18½ hours very faint pink; (8) very like (7); (9) again similar but a little bluer and already blue in very thin layers, after

8 minutes became weak grey violet, after 18½ hours almost colourless; (10) much bluer violet and pure blue in thin layers, after 18½ hours extremely weak grey; (11) similar to (10) but less dichroic, not so red violet in thicker layers, after 18½ hours same as (10); (12) same as (11); (13) same as (11), after 18½ hours pale orange yellow; (14) less dichroic than (11), after 18½ hours more intensely yellow than (13); (15) still less dichroic and still deeper yellow on keeping 18½ hours; (16) greenish blue, after 30 minutes colour largely disappeared, after 18½ hours deeper yellow than (13); (17) bluish green, after 8 minutes almost colourless, after 30 minutes orange yellow, after 18½ hours deeper yellow than (13).

5-O-*Benzoylcyanidin chloride.* The alcoholic solution was the colour of an aqueous permanganate solution. 1 % hydrochloric acid, bluish red of no great tinctorial intensity, after 30 minutes faint pink, after 1½ hours colourless; 20 % hydrochloric acid, the salt was precipitated leaving a colourless solution; (1) bluish red rapidly fading and colourless after 1 minute; (2) bluish red, after 12 minutes colourless; (3) similar but still slower fading, after 12 minutes faint pink, after 30 minutes colourless; (4) similar, a little slower fading; (5) red violet, after 12 minutes weak violet pink, after 30 minutes pale reddish violet, after 24 hours colourless; (6) more intense violet fading somewhat rapidly, after 12 minutes very pale violet, after 24 hours still a violet tinge; (7) very similar to (6) but faded a little less throughout; (8) similar to (7) and fading a little less throughout; (9) blue violet, did not fade very rapidly but became more violet; (10) blue with violet tinge becoming more violet, after 12 minutes violet blue, after 30 minutes violet, after 24 hours reddish yellow; (11) blue with slight violet tinge, subsequent changes like (10); (12) pure blue in thin layers, after 6 minutes violet blue, after 25 minutes blue violet, after 24 hours yellow; (13) same as (12) but became violet a little more slowly; (14) pure blue, after 20 minutes green, after 24 hours yellow; (15) pure blue, after 5 minutes green, after 20 minutes greenish yellow, after 24 hours yellow; (16) greenish blue changing through green to yellow more quickly than (15); (17) similar to (16).

Peonidin chloride. 1 % and 20 % hydrochloric acid, eosin red, same shade as cyanidin, unchanged after 18½ hours; (1) eosin red a little bluer than hydrochloric acid solutions, after 15 minutes faded and after 24 hours colourless; (2) cherry red, fading is most rapid at this p_H, after 30 minutes colourless; (3) bluer red, after 2 minutes browner red fading rapidly, after 15 minutes the colour was weak, after 11½ hours colourless; (4) bluish red which quickly faded and became browner, after 18½ hours some colour-base had separated; (5) very much like (4); (6) reddish violet which did not fade so quickly as (5), (4), (3) and (2), after 18½ hours some colour-base had separated; (7) violet with reddish tinge, bluer than (6), fading comparatively slowly, after 18½ hours faint pink and no precipitate; (8) violet, after 18½ hours faint reddish violet; (9) bluish violet, blue in thin layers, after 18½ hours weak violet; (10) blue, violet in thick layers, after 18½ hours bluish grey; (11) a little greener blue

becoming still greener in 1 minute, after 18½ hours bluish grey; (12) like (11), after 18½ hours greenish grey; (13) greenish blue, after 18½ hours weak yellow; (14), (15), (16), (17) almost same as (13) but curiously the fading in (16) and (17) was less rapid than in (14) and (15); this suggests that both oxidation and *pseudo*-base formation are involved.

Malvidin chloride. 1 % and 20 % hydrochloric acid bluish red, unchanged after 24 hours; (1) a little bluer red, weak pink after 20 minutes, colourless after 50 minutes; (2) similar to (1) but fading more rapidly, almost colourless after 20 minutes; (3) bluish red at once changing to pale brownish cherry red, after 5 minutes pale rose, almost colourless after 20 minutes; (4) similar to (3); (5) similar to (3) and (4) at first but fading is most rapid and in less than 15 minutes there was a brown violet red precipitate in a colourless solution, after 3 hours there was still a flocculent precipitate but after 5 hours all the solid had passed into solution; (6) violet fading rapidly with a slight precipitation; almost colourless after 3 hours; (7) pure violet solution blue in thin layers and no precipitate, after a few minutes faded violet blue, after 3 hours pale blue, after 24 hours almost colourless; (8) blue, violet in thick layers and rapidly fading, after 10 minutes the series (8), (9), (10), (11), (12) had all faded similarly and there was little divergence in the colours which ranged from weak violet blue to weak greenish blue, after 2½ hours the series (8) to (12) were very pale blue to greenish blue, after 24 hours (10), (11) and (12) were pale orange; (9) pale blue fading rapidly; (10) greenish blue fading rapidly; (11) similar to (10); (12) similar to (10); (13) greenish blue becoming greener as it faded, bluish green after 30 minutes, the change to green occurred more and more rapidly in the ensuing series (13) to (17), on keeping changes like (10); (14) similar, yellowish green after 1½ hours; (15) similar, greenish yellow after 1½ hours; (16) similar, brownish yellow after 1½ hours; (17) similar.

Cyanin chloride. In the case of anthocyanins, 25·00 mg. were dissolved in 100 cc. of methyl alcoholic hydrochloric acid (1 cc. aqueous hydrochloric acid, sp. gr. 1·16, made up to 1000 cc. with methyl alcohol); the acid thus introduced causes a reduction of 0·1 in the p_H of all the solutions. The specimen of cyanin chloride employed was derived from deep-red dahlia flowers and was kindly sent to us by Professor R. Willstätter, to whom we are greatly indebted.

Solutions in 1 % and 20 % hydrochloric acid were salmon red; (1) pink, much less intense owing to *pseudo*-base formation; (2) a little bluer than (1) and rapidly faded; (3) bluish red fading rapidly; (4) permanganate-coloured fading rapidly; (5) similar, fading less quickly; (6) similar but colour more intense and fading quite slowly; (7), (8), (9) similar to (6); (10) reddish violet; (11) violet; (12) violet; (13) bluer violet; (14) violet blue fading quickly; (15) blue fading quickly; (16) greener blue fading rapidly; (17) similar.

After 15 to 20 minutes (1) was pink and the colour diminished in the series to (5) which was colourless; (6) had faded but was intense in comparison with (1); (7), (8), (9), (10), (11) and (12) had faded somewhat and formed

a series ranging from violet red to violet; (13) was weak violet; (14) weak violet blue and (15), (16) and (17) were almost colourless (grey).

After 5 hours (1) was feebly pink and the colour diminished to (5) which was colourless, then gradually increasing reddish violet to (10) which was however very pale coloured; (11) was similar but not so clear toned; (12) to (17) inclusive were pale yellow.

After 20 hours the solutions in 1 % and 20 % hydrochloric acid were unchanged; (1) was pink and the colour in the range diminished to (5) which was colourless, then increased to (9) which was weak pink; (10) was weak brownish pink and (11) and the remainder were pale yellow.

Malvin chloride. We are greatly indebted to Professor P. Karrer for the provision of a specimen of this diglucosidic anthocyanin. The solutions in 1 % and 20 % hydrochloric acid were bluish red; (1) bluish red becoming pale rose in a few seconds; (2) quickly decolorised; (3) same as (2); (4) bluish red fading in the course of 2 minutes; (5) reddish violet fading slowly; (6) violet fading slowly; (7) bluish violet; (8) violet blue; (9) blue; (10), (11) and (12) similar to (9); (13) blue fading to greenish blue; (14), (15), (16), (17) blue fading with increasing rapidity to pale green.

After 20 minutes (1) was pink, (2), (3), (4) and (5) were colourless, (6) was weak reddish violet, (7) was a little deeper violet; (8) was bluish violet; (9), (10), (11) and (12) were blue; (13) was greenish blue; (14) was bluish green; (15) was yellowish green; (16) was greenish yellow, and (17) was yellow.

After 5 hours (1) to (8) inclusive were almost colourless ((1) was tinged pink, (7) reddish violet, and (8) pale blue); (9) was weak violet blue; (10) was weak pure blue; (11) was pale greenish blue; (12) was pale green, and (13) to (17) inclusive were yellow.

After 20 hours (1) was faint pink; (2) to (6) inclusive were colourless; (7) was very faint pink; (8) was very faint blue; (9) was pale blue; (10) was blue; (11) was bluish green, and (12) to (17) were yellow. The solutions in 1 % and 20 % hydrochloric acid were unchanged.

Other anthocyanins have been similarly characterised and the results will be subsequently recorded in connection with synthetical experiments.

It should be stated that all the tests were carried out in the summer and that the room temperature was 20–25°; no precautions were taken to exclude air.

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VII. THE RELATION BETWEEN CYSTINE YIELD AND TOTAL SULPHUR IN WOOL.

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In an investigation now in progress upon the nature of the sulphur linkage in wool, it became important to know whether the whole of the sulphur could be accounted for as cystine, or whether there existed other, hitherto undiscovered, amino-acids present in the protein molecule and containing the element sulphur in their constitution. Suggestions of such a nature have been made from time to time.

The kind of evidence usually brought forward as indicative of the presence of sulphur-containing substances other than cystine is divisible, broadly, into arguments of two kinds. First, differences have been noted and emphasised in the manner of reaction, towards alkaline solutions, of cystine and proteins. The latter lose all or a part of their sulphur with comparative ease, the sulphide ion being formed; cystine solutions under similar conditions suffer far less change. Moreover the fraction of the total sulphur which is eliminated as sulphide is not the same in all proteins but varies between wide limits [see Osborne, 1900; Fleitman, 1847, 1848; Krüger, 1888; Mörner, 1901].

The second class of arguments was based on the fact that in no case does the yield of cystine obtained from a protein after hydrolysis approach the value to be expected, were its sulphur present solely in this form. This aspect of the matter has been dealt with by Harris [1923] and by others. Cystine is not an easy amino-acid to isolate in quantitative yield, even from pure solutions, as Harris has shown, and its solubility, and therefore the difficulty of its separation, is enormously increased by the presence of other amino-acids [cf. Hopkins, 1921].

The methods available for its isolation have been reviewed by Barritt [1927], whose work represents the most recent attempt to obtain quantitative isolation from wool. Even under the most suitable conditions, in no case was he able to obtain more cystine than corresponded to 66 % of the total sulphur.

Since the work of Abel and his associates upon the sulphur of insulin, the question of the ratio of "labile" to "firmly bound" sulphur in proteins has taken on a new interest. In this connection Brand and Sandberg [1926] have

made the important observation that lability of the sulphur atom towards alkali differs enormously in the different compounds of cystine. Results of a similar kind have been obtained by Bergmann and Stather [1926]. On the other hand, du Vigneaud, Jensen and Wintersteiner [1928], working with crystallised insulin, found that after acid hydrolysis only about one half of the total sulphur could be accounted for as cystine, using the Sullivan [1926] colorimetric method for the determination of the latter, whilst Folin and Looney's [1922] colorimetric reagent gave a value consistently higher and approximating closely to that of the total sulphur. The Sullivan method is—so far as is known—specific for cystine, whilst the Folin and Looney reagent develops a colour with all substances in which the S—S linkage is present. Hence it seemed probable to du Vigneaud and his collaborators that there was present in insulin a substance which was not cystine but which contained sulphur in disulphide linkage.

Accordingly, for the purposes of the present investigation, it was decided to carry out cystine determinations upon acid hydrolysates of wool samples of different origin, using the two colorimetric methods mentioned above, and to compare the results so obtained with the cystine content reckoned by assuming all the sulphur of the wool to be present as cystine.

In every case examined a very close agreement has been found between these two values, all the sulphur of the wool being accountable as cystine measured by the Sullivan method (the most specific, short of direct isolation). The Folin and Looney values were found consistently to be slightly higher than the Sullivan values—and higher than the total sulphur—but this reagent is known to give a blue colour with tyrosine, hydroxytryptophan and other amino-acids.

The conclusion is drawn, therefore, that the whole of the sulphur of wool is liberated as cystine when the protein is hydrolysed by acid. The method is clearly applicable to all other proteins.

Experimental.

Raw wool samples were used in every case. These were degreased by washing twice in warm (50°) benzene, air-dried, and then washed thoroughly in cold 0·1 % saponin solution. Washing was effected by rinsing in many successive changes of distilled water, using a relatively large volume for each rinse. The wool was again air-dried by leaving it spread out very thinly in a warm dry atmosphere for several days. Samples were then taken for sulphur and moisture (regain) determination and at the same time for hydrolysis. Moisture content was determined by recording the loss in weight when a current of dry air was led over the sample contained in a specially designed drying bottle and held at a temperature of 105°. Total sulphur was determined by the Benedict-Denis method as used by Trotman and Bell [1926], or by the Carius method.

For hydrolysis 6 g. of air-dried wool were weighed out and added to 100 cc. of boiling 20 % hydrochloric acid. Boiling was maintained, using a sand plate and reflux condenser and samples of 5 cc. were withdrawn at intervals of $\frac{1}{4}$, $\frac{1}{2}$ hour, and thence at every hour from the time of commencement. Each sample was pipetted into a 50 cc. volumetric flask, diluted largely with water, and then rendered nearly neutral by the cautious addition of 5 cc. of 20 % sodium hydroxide, after which the total volume was made up to 50 cc. and the sample filtered. For cystine determination according to the Sullivan method 2 cc. of the sample were used, and a suitable amount of a standard 0·05 % cystine solution in 0·1 *N* hydrochloric acid, water being added to the standard if necessary to bring the volume to 2 cc. To each, 1 cc. of a 5 % sodium cyanide solution was added and after exactly 10 minutes 1 cc. of a 0·5 % solution of 1:2-naphthoquinone-4-sodium sulphonate and 5 cc. of 10 % sodium sulphite in 0·5 *N* sodium hydroxide. After 15 minutes each flask received 1 cc. of 2 % sodium hydrosulphite in 0·5 *N* sodium hydroxide. Colours were compared in a Bausch and Lomb colorimeter. It was found by experience that results were not dependable unless standard and unknown solutions contained very nearly the same quantity of cystine, and therefore a separate standard was made up for determination of each sample, allowing a difference in reading of not more than 15 %. The sulphite, hydrosulphite and quinone solutions were always freshly prepared, never being more than four hours old.

Using these precautions the method gave very satisfactory results. Since long boiling of cystine with strong acids converts it into an isomeric form differing from true cystine in crystalline form and in being optically inactive [Hoffman and Gortner, 1922], it was thought necessary to prepare some of this isomeric cystine and ascertain whether it was estimable by the Sullivan method. Accordingly 10 g. of cystine were boiled under reflux with 150 cc. of 20 % hydrochloric acid for 144 hours. The solution was filtered, decolorised with charcoal and the cystine precipitated by bringing to p_H 3·7 with sodium acetate. After recrystallisation it was analysed and compared with ordinary cystine by the Sullivan method. It gave 99·5 % of the colour of true cystine, an agreement which is well within the sensitivity of the method.

The Folin and Looney method was carried out employing 2 cc. of the test solutions and 2 cc. of the cystine standard. To each, 3 cc. of water were added, followed by 5 cc. of *N* sodium hydroxide and 10 cc. of fresh 20 % aqueous sodium sulphite. After 5 minutes, 3 cc. of the Folin and Looney reagent were added, and after waiting another 10 minutes the solutions were made up to a total volume of 25 cc. and compared.

The cystine used as standard was analytically pure (S = 26·79 %).

Total sulphur was determined upon 5 cc. of the hydrolysate by evaporating the hydrochloric acid in a silica dish on the water-bath adding 5 cc. of Benedict-Denis reagent and proceeding in the usual manner.

A typical result is shown in the curve, Fig. 1 (Crossbred 50's wool). The

liberation of free cystine proceeds rapidly and is complete within 5 to 7 hours. There is some indication of a fall after the maximum cystine value has been reached. This is in agreement with the findings of Merrill [1921], who also obtained the greatest yield of cystine from wool after 5 to 7 hours' hydrolysis. This destruction of cystine probably accounts for the fact that the maximum value is generally about 0·5 % short of the total sulphur figure reckoned as cystine. It hardly seems likely that any other amino-acid should be responsible for this very slight amount of extra sulphur. The values obtained by the Folin and Looney method correspond closely with the maximum Sullivan value with the exception of the first three or four points (1 to 2 hours). The fall from a value some 10 % higher than the final steady line is a constant feature observed in every experiment and can only be interpreted as indicating the formation of substances (possibly of phenolic nature) to which the reagent is

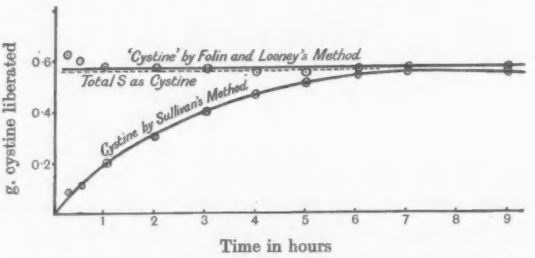

Fig. 1. Hydrolysis of wool (Crossbred 50's) by 20 % HCl.

sensitive and which are comparatively rapidly destroyed. Abderhalden and Fuchs [1913] have shown that tryptophan and hydroxytryptophan give a blue colour with the phosphomolybdate-tungstate reagent; they may equally well react in the present circumstance. It is well known that these substances are not stable under the conditions of an acid hydrolysis. The Folin and Looney values, it will be noticed, do not show a gradual increase in cystine as hydrolysis proceeds, but reach their maximum directly the wool is dissolved (within 10 minutes). As the reagent reacts with the disulphide linkage it would naturally react with cystine peptides and hence gives the form of curve to be expected.

Most of the preliminary work was done upon a Crossbred 50's wool, S = 3·46 %, but among other samples examined were a typical low sulphur wool—Devon lamb's wool, S = 3·34 %, and a high sulphur wool—Welsh mountain[1], S = 4·08 %, and also a fine and a coarse Turkey mohair. No difference in behaviour was found between these and other wools. The following table shows the results obtained.

[1] For these samples and the determination of their total sulphur contents, I am indebted to Mr Barritt, of this Association.

Table I.

Wool	Total S % dry wt.	Cystine in hydrolysate: By Sullivan's method (max. value) g.	Cystine in hydrolysate: By Folin and Looney's method g.	Total S of hydrolysate (as cystine) g.	Ratio*
Crossbred 50's I	3·46	0·3780	—	0·3602	+4·94
" " II	3·47	0·5700	0·5950	0·5815	−1·99
" " III	3·54	0·5575	0·5673	0·5589	−0·25
Devon lamb	3·34	0·5755	0·5825	0·6001	−4·10
Welsh mountain	4·08	0·6300	0·6400	0·6277	+0·37
Turkey mohair (coarse)	3·02	0·5978	0·6080	0·5948	+0·50
Turkey mohair (fine)	3·18	0·5405	0·5564	0·5393	+0·22
Cape merino	3·67	0·5875	0·5963	0·5887	−0·02

* $\frac{\text{Total S (hydrolysate)} - \text{cystine (Sullivan)} \times 100}{\text{Total S}}$.

Precipitation of cystine by phosphotungstic acid.

Since du Vigneaud, Jensen and Wintersteiner [1928], found that their non-cystine, sulphur-containing substance could be approximately separated from cystine by precipitation of the latter with phosphotungstic acid, it was decided in the present case to adopt a similar procedure in order to check the results obtained.

40 cc. of an acid hydrolysate—containing in all 232 mg. of cystine—were diluted to 240 cc. and 15 g. of phosphotungstic acid added. The flask was stoppered and immersed in ice for 24 hours. The phosphotungstates were then filtered off, washed and dissolved in sodium hydroxide. Phosphotungstic acid was removed from this solution and from the filtrate by barium chloride in the usual way and excess of barium by sulphuric acid. Determinations were then carried out by the Sullivan and Folin and Looney methods and yielded the following results.

	Phosphotungstate precipitate	Filtrate
Total cystine by Sullivan method. 117·5 mg.... ...		55·0 mg.
Total cystine by Folin and Looney method. 120·6 mg.		65·0 mg.
Total tyrosine by Folin and Ciocalteu method [1927]		100·8 mg.
Equivalent colour as cystine[1]		9·4 mg.

Wool contains about 3 % of tyrosine. The quantity present in this experiment gives an equivalent cystine value with the Folin and Looney reagent of 9·4 mg. in the filtrate. Tyrosine alone would therefore account almost entirely for the discrepancy observed between the figures in the last column. A second experiment gave similar results.

Determination of sulphur in wool from cystine yield.

From the quantity of cystine formed when a sample of wool is boiled with hydrochloric acid, the amount of sulphur originally present in the wool may

[1] 10 mg. tyrosine were found to give the same colour with the Folin and Looney reagent as 0·93 mg. of cystine.

be determined. The following example is taken from an actual experiment using a Crossbred 50's wool.

A known weight of wool was boiled for 7 hours in a Kjeldahl flask with 20 % hydrochloric acid. When cool, the hydrolysate was neutralised by sodium hydroxide, the solution filtered and, together with the washings, made up to 100 cc. Cystine was determined in 2 cc. of this liquid using the Sullivan method.

The moisture content of the wool was ascertained by weighing out a sample at the same time that the sample for hydrolysis was taken and proceeding as described earlier in this paper.

The following figures were obtained.

Weight of wool hydrolysed	0·5183 g.
Moisture content	13·27 % of wet wt.
Cystine in acid hydrolysate	57·75 mg.

whence

Sulphur content of dry wool	3·43 %.

The sulphur content by the Carius method was found to be 3·46 %.

Summary.

It may be concluded from these experiments that natural wools differing markedly in their sulphur content (3·34 % to 4·08 %—nearly the extremes of variation encountered) and also fine and coarse samples of Turkey mohair are all capable of yielding their entire sulphur as cystine when hydrolysed by acids. There is no evidence of any other sulphur compound entering into their constitution.

The author's acknowledgments are made to Mr A. T. King, Chief Chemist to the Association, for help and advice rendered during the course of the work.

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VIII. A SPECIFIC COLOUR REACTION FOR ERGOSTEROL.

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The unique function of ergosterol as the parent substance of vitamin D made it desirable to find a colour reaction for it, by means of which it could be detected in the presence of other sterols. In searching for such a reaction, the property of formaldehyde of shifting the colour from the red into the blue part of the spectrum in the usual colour reactions of sterols [Whitby, 1923; Rosenheim, 1927] suggested an investigation of the behaviour towards sterols of the aldehyde corresponding to trichloroacetic acid. This acid is known to give rise to colour reactions with cholesterol [Tschugajeff, 1900].

Although anhydrous chloral proved to be non-reactive, it was found that both chloral hydrate and trichloroacetic acid give a characteristic blue colour reaction with ergosterol, whilst all the other naturally occurring sterols investigated, when purified from ergosterol, remain colourless under the same conditions. The specimens of ergosterol used in these experiments had been fractionally recrystallised at 36° and possessed the highest optical activity, $[\alpha]_D - 132°$, so far recorded [Tanret, 1908; see also Bills and Honeywell, 1928]. The reactions to be described are therefore unlikely to be due to impurities or degradation products of ergosterol.

In contradistinction to naturally occurring sterols it was found, when studying the reaction of sterol derivatives, that the production of an immediate red colour with either of these reagents is specific for the $\Delta^{1,2}$ (or $\Delta^{1,13}$) linkage of the sterol ring system. These observations suggest an explanation of the mechanism of sterol colour reactions, which is applicable to all of them and will be discussed later.

I. *Chloral hydrate reaction.*

When a few crystals, 1 mg. or less, of ergosterol are added to about 0·5 g. chloral hydrate, liquefied by warming in a water-bath, they dissolve and immediately give rise to a carmine red solution, showing a broad absorption band at 500 $\mu\mu$. The red colour changes within a minute into a green and finally into a deep blue, which persists for a considerable time. The esters of ergosterol react in the same way. The colour is discharged rapidly

by water or alcohol, more slowly by chloroform, benzene, toluene or other anhydrous solvents not possessing a hydroxyl group.

Although the blue colour is discharged by dilution with water, a saturated aqueous solution of chloral hydrate (80 %) reacts in the typical manner when a drop of concentrated HCl is added. It would therefore appear that traces of an acid are essential for the reaction.

Freshly distilled anhydrous chloral dissolves ergosterol. The colourless solution undergoes the above-described changes on the addition of one drop of water. On keeping under laboratory conditions, chloral, or its chloroform solution, attracts moisture and gradually becomes "activated," so as to give the reaction with ergosterol without the addition of water.

Colourless solutions are given under these conditions by all the other naturally occurring sterols, their esters and their reduction products. The following carefully purified sterols were examined: cholesterol, sitosterol, γ-sitosterol (1 double bond); stigmasterol (2 double bonds); zymosterol, fungisterol (3 double bonds); *iso*cholesterol (saturated?), amyrol, coprosterol and dihydrositosterol (saturated). In the preparation of zymosterol from yeast and fungisterol from ergot [Rosenheim and Webster, 1928], it was found that the crude specimens gave the reaction strongly. The intensity of the reaction decreased, however, proportionately to their progressive purification and the removal of ergosterol. The agreement of the spectroscopic and biological examination with the colorimetric results justifies the conclusion that the slight positive reaction of the purest specimens of zymosterol and fungisterol obtained so far is due to admixture of ergosterol.

II. *Trichloroacetic acid reaction.*

An aqueous solution, prepared by dissolving nine parts of the pure crystallised acid in one part water, was found to be the most suitable reagent. When this is added to ergosterol dissolved in a few drops of chloroform, an immediate red solution (band at 500 $\mu\mu$) is produced, which changes gradually into a clear blue (bands at 570–580 and at 650–680 $\mu\mu$), without showing the intermediate green phase of the chloral hydrate reaction. In distinction from the latter, the reaction takes place at ordinary temperature and has the further advantage of yielding a final blue solution, which may be diluted for colorimetric purposes with the reagent itself or with chloroform. Excessive dilution with chloroform or other solvents tends to change the colour into a bluish-green of the same shade as that given by ergosterol with a saturated chloroform solution of trichloroacetic acid. The latter solution undergoes decomposition on keeping, liberating phosgene and hydrochloric acid, and is not so suitable a reagent as the aqueous solution.

The sensitiveness of the reaction was determined by adding three drops of the reagent to 0·1 cc. of a chloroform solution containing known amounts of ergosterol. It was found that 0·01 mg. ergosterol still gave a marked reaction

within 5 minutes, and that the colour is just recognisable with 0·005 mg. when compared with the colourless control. The sensitiveness is therefore of approximately the same order as that of the usual sterol reactions.

Employing mixtures of cholesterol and ergosterol, prepared by mixing their solutions in chloroform, a strong reaction is obtained in the presence of 0·5 % ergosterol in 0·1 g. cholesterol, and even smaller quantities down to 0·1 % can be detected by comparison with the colourless cholesterol control. The amount of ergosterol in ordinary cholesterol, as estimated by the spectroscopic test, may vary from 0–0·1 % according to the method of purification used and depending on the source of the sterol. Cholesterol prepared from brain or cod-liver oil, having undergone charcoal treatment, may occasionally be free from ergosterol [Rosenheim and Webster, 1927], whilst as much as 0·12 % may be present in preparations from spinal cord [Bills, Honeywell and MacNair, 1928]. Examination of a large number of "pure" specimens, M.P. 147–148°, prepared from brain, gallstones, liver, spleen, skin, blood, ovaries (pig), eggs (frog) and cod-liver oil gave negative tests in some cases (brain, gallstones) and positive reactions in most. The intensity of the reaction in descending order was approximately: eggs (frog), ovaries, liver, skin (pig), brain, spleen, blood. Specimens of sitosterol from wheat and maize also gave positive reactions before purification by the bromine method. Whilst the colour reaction for ergosterol in sterols probably does not equal that of the biological or spectroscopic test in sensitiveness, it affords chemical evidence for the assumption that the impurity in ordinary cholesterol, which gives rise to vitamin D on irradiation, is identical with ergosterol.

The other naturally occurring sterols examined (see above) do not react with the freshly prepared reagent and remain colourless for more than 30 hours when kept at room temperature in the dark. On warming, however, cholesterol [Tschugajeff, 1900; Hirschsohn, 1902] and the other unsaturated sterols rapidly give a red solution, showing an absorption band at 500 $\mu\mu$.

A specific reaction for the $\Delta^{1,2}$ *(or* $\Delta^{1,13}$*) linkage in sterols.*

A study of the behaviour of the two reagents towards cholesterol derivatives, in which known chemical changes had been produced, showed that esterification of the hydroxyl group, its replacement by chlorine, or its complete removal, has no effect, for cholesterol acetate or chloride and cholestene gave colourless solutions. Reduction or bromination, etc., of the double bond are equally without influence, since dihydrocholesterol, cholestane, cholesterol dibromide and cholesterol oxide are also non-reactive. The doubly unsaturated ketone, "oxycholesterilene" and dicholesteryl ether, as well as digitaligenin, also gave negative results.

On applying the chloral hydrate and the trichloroacetic acid reaction to *allo*cholesterol and *allo*sitosterol, however, an immediate yellow → orange colour was produced, which rapidly deepened into a permanent carmine red. In both cases the red solutions showed an absorption band at 500 $\mu\mu$. In

the $AsCl_3$ and $SbCl_3$ reactions, the behaviour of the *allo*-compounds is identical with that which they show with the above reagents.

The conversion of cholesterol into the isomeride *allo*cholesterol was effected by means of hydrochloric acid by Windaus [1927], who also showed conclusively that a shifting of the double bond from $C_{6,7}$ to $C_{1,2}$ (or $C_{1,13}$) takes place under these conditions (see formulae I and II, p. 52).

The above results indicate that an immediate red colour reaction is directly dependent on the presence of the $\Delta^{1,2}$ linkage[1], and it seemed therefore of interest to test the behaviour of other sterol derivatives, in which the existence of the same linkage had been postulated. Heilbron and Sexton [1928] arrived at the conclusion that "one of the ethenoid linkages in cholesterilene must occupy the same position as in ψ-cholestene," which latter substance possesses only one double linkage, presumably in the same position as in *allo*cholesterol. Specimens of cholesterilene, ψ-cholestene and ψ-cholestane, prepared by these authors, were kindly put at my disposal by Prof. Heilbron, and it was found that both cholesterilene and ψ-cholestene gave an intense red reaction, the latter somewhat more slowly than the former. On the other hand, ψ-cholestane remained colourless, as was to be expected in the case of this saturated hydrocarbon.

The positive results confirm Heilbron and Sexton's conclusions, and may be taken as supplementary evidence for the specificity of the reaction. They lead further to the suggestion that the primary red phase of the ergosterol reaction (see above) is due to the presence of the $\Delta^{1,2}$ linkage. This conclusion is in agreement with that reached by Heilbron, Morton and Sexton [1928] from their studies of the ultra-violet absorption spectrum of cholesterilene, which led them to infer "that of the three double bonds in ergosterol, two occupy the same position as in cholesterilene." The final blue stage of the ergosterol reaction may therefore justifiably be ascribed to the influence of the third double linkage, the position of which is at present unknown.

It is of interest to note further that an immediate red reaction is also given by β-cholesterol, an isomeride of cholesterol obtained by Diels and Abderhalden [1908], by heating cholesterol to 310°. The position of the double bond in this isomeride remains unknown. It seems permissible to infer from the positive result of the above colour reaction that β-cholesterol also contains the $\Delta^{1,2}$ linkage, its isomerism with *allo*cholesterol being due to the attachment of the hydrogen atom to the carbon atom C_2 (or C_{13}) in either the *cis*- or *trans*-position.

The bathychromic effect of formaldehyde. In two colour reactions of cholesterol, namely with concentrated H_2SO_4 and with $AsCl_3$, formaldehyde has a bathychromic effect, *i.e.* it deepens the colour from red to violet [Whitby, 1923; Rosenheim, 1927]. The same effect is observed in the trichloroacetic acid reaction with *allo*cholesterol and *allo*sitosterol: if a few drops of formalin

[1] For convenience of discussion, the symbol $\Delta^{1,2}$ only is used in the following, implying also the possibility of the $\Delta^{1,13}$ linkage.

are added when the red phase has been reached, the colour changes gradually into a purple and violet. The reaction is conveniently carried out with a saturated solution of the acid in formalin as a reagent, which produces rapidly a violet reaction with the *allo*-compounds, showing a band at 590–610 $\mu\mu$. Cholesterol behaves in the same way with trichloroacetic acid and formalin, but heating in this case is necessary [Golodetz, 1908]. In the reaction with ergosterol, on the other hand, formaldehyde exerts a hypsochromic effect, and prevents the formation of the blue phase. When added to the reaction mixture at the blue stage, it gradually weakens and discharges the colour.

It has been shown previously [Rosenheim, 1927] that, on heating cholesterol in chloroform solution with benzoyl peroxide, the white product obtained gives a blue colour with the reagents which usually give rise to a red colour. When similarly treated, *allo*cholesterol also gives a blue colour with trichloroacetic acid. The gentian-blue colour of this reaction is, however, easily distinguished by the naked eye from the colour of the ergosterol reaction (starch-iodine-blue), and spectroscopic examination shows in the former solution the characteristic band of "oxycholesterol" only. The blue reaction of "oxycholesterol" may simulate the presence of ergosterol in specimens of cholesterol which have been exposed to light or ultra-violet irradiation in presence of air, but is easily differentiated by its absorption band from the ergosterol reaction[1].

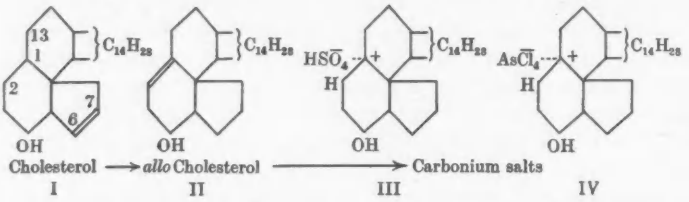
The mechanism of sterol colour reactions.

Various attempts have been made to find an explanation for the numerous sterol colour reactions, which are obviously due to one common factor. Mauthner [1909] considered the presence of double linkages as essential, since the reactions are negative with completely reduced derivatives of cholesterol. In explanation of Liebermann's reaction (acetic anhydride and concentrated H_2SO_4) Wieland and Weil [1913] suggest that the coloured substances are halochromic sulphates of ketones, the latter being produced by the action of acetic anhydride on a reactive double linkage. On the other hand, Whitby [1923] assumes that the sterol colours are produced by the condensation of an aldehydic coupling substance (*e.g.* formaldehyde) with a hydrocarbon (*e.g.* cholesterilene), both of which are presumably formed from the sterol by the condensing agent used (*e.g.* sulphuric acid). There appears to be, however, no evidence for the formation of the postulated aldehydic coupling substance under the conditions of the test, and on other grounds also Whitby's explanation has been criticised [Wokes, 1928].

The reactions described above, which show that an immediate red colour reaction is given only by those sterols or their derivatives which contain the $\Delta^{1,2}$ linkage, suggest a general explanation for the chromogenic properties of sterols. According to this, the primary reaction consists in the isomerisation

[1] The oxidising action of charcoal also gives rise to "oxycholesterol" formation. [Blix and Löwenhielm, 1928.]

of the sterol, under the influence of a strong acid, giving rise to an isomeride containing the $\Delta^{1,2}$ linkage. In these compounds (II) the carbon atom C_1 has become doubly linked. Owing to its position at the junction of two rings, C_1 is assumed to have acquired a property similar to that of the tervalent carbon atom in triphenylmethyl, and to be able, like the latter [Baeyer, 1905, *int. al.*] to form carbonium salts, which are coloured (III and IV).



This assumption is supported by the fact that the sterol colours are discharged by water or alcohol, and that in their formation strong acids are used (*e.g.* concentrated H_2SO_4 in Salkowski's, Liebermann's, Whitby's, etc. reactions), or generated from the reagent (HCl from $AsCl_3$ in Kahlenberg's reaction). In the latter case, as well as in the trichloroacetic acid reaction, it is significant in this connection that a rapid colour change with sterols other than *allo*cholesterol, etc., requires heat, which also favours isomerisation: a solution of cholesterol in $AsCl_3$ or trichloroacetic acid remains colourless when kept at 0°. The identity of the absorption band in all these reactions with that of *allo*cholesterol is a further indication of the similarity in constitution of the coloured substances formed.

Summary.

1. Ergosterol gives a blue colour reaction with chloral hydrate and with trichloroacetic acid, by means of which it may be detected in the presence of other naturally occurring sterols.

2. An immediate red colour reaction with the above reagents is specific for those sterol derivatives which possess the $\Delta^{1,2}$ (or $\Delta^{1,13}$) linkage.

3. It is suggested that the primary reaction in all sterol colour reactions consists in the shifting of the double linkage into the $C_{1,2}$ (or $C_{1,13}$) position and the subsequent formation of coloured carbonium salts.

I am indebted to Dr Anderson for specimens of natural dihydrositosterol and γ-sitosterol, to Prof. Drummond for a specimen of *iso*cholesterol from lanolin, and to Messrs Hoffmann-La Roche & Co. and Messrs Boots, Ltd. for zymosterol.

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IX. THE VITAMIN CONTENT OF HONEY.

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Report to the Medical Research Council.

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The experiments recorded in the literature suggest that honey is not a good source of vitamins. Dutcher [1918] tested strained honey from newly-filled combs, obtained while clover and basswood were in bloom, on pigeons and came to the conclusion that the "water-soluble B" content of the product was negligible. Faber [1920] found that daily doses of 4–8·4 cc. of white sage comb honey bought in the open market failed to protect guinea-pigs from scurvy. Hawk, Smith and Bergheim [1921] were also unable to detect vitamin A, B or C in strained honey, as were Scheunert, Schieblich and Schwanebeck [1923] using a centrifuged lime honey from East Prussia, a comb honey with wax from Lüneberger Heide and a honey not of German origin[1]. Great progress has been made in the domain of vitamin research since the above tests were carried out. This communication records the tests for vitamins A, B_1, B_2, C and D on two representative samples of honey as carried out by the more refined methods now available.

Experimental.

One of the samples was a granular honey containing 82·23 % of dry matter and 0·17 % of ash of West Indian origin kindly supplied by Sir Algernon Aspinall. The other sample was an English honey prepared at the Bee Research Institute, Rothamsted, under the supervision of Mr D. Morland, M.A., to whom I should like to express here my indebtedness for the help he extended. The following is Mr Morland's report of the conditions under which the honey was produced.

"The honey was gathered by a stock of Italian bees at Kimpton Hoo, near Welwyn, Herts. A clean super of new frames with wax foundation was given to the colony on July 12th, 1928. At that time the colony had brood in two chambers (British Standard) and had partly filled one super with honey. No queen excluder was used. The weather was favourable throughout the period. The sunshine figures at Rothamsted, which is a few miles from Welwyn, for the period July 12th to July 28th inclusive show a total of 173·8 hours of 'bright sunshine.' White clover was yielding at the time. Much of the neighbourhood is park grassland and the subsoil is chalk. The super was removed

[1] According to the Annual Bureau of Home Economics (1927) two examples of honey examined were found deficient in vitamins A, B, C and D (quoted by Phillips in *Gleanings from Bee Culture*, Jan. 1929).

on July 28th. The hive had increased in weight during this period from 153¼ lb. to 232½ lb. The combs used in the feeding experiment were fully sealed over and the honey appeared to be of excellent quality."

This honey when extracted contained 84·95 % of dry matter and 0·10 % of ash.

In order to extract the English honey from the comb the super was mounted horizontally and the sealings of the cells were removed with a hot knife. The wax and honey of these exposed cells were then removed by means of a spoon and filtered through muslin which retained the wax completely. Just sufficient honey for the day's dosing was extracted daily. The West Indian honey was fed without previous treatment.

Vitamin A.

Vitamin A was estimated by a method based on the Steenbock principle. Young rats weighing 35–40 g. were placed in separate cages on a diet free from the fat-soluble vitamins [Zilva and Miura, 1921]. At the end of 28 days the ordinary cotton-seed oil of the basal diet was replaced by irradiated cotton-seed oil. When the cessation of growth due to the deficiency of vitamin A became apparent, 2 g. of honey were administered daily previous to the delivery of the basal diet. With each test a series of negative controls (on the basal diet alone) and of positive controls receiving 3 drops of cod-liver oil daily were employed. It will be seen from Fig. 1 that the growth curves of the rats which received the honey are similar to those of the negative control animals and consequently a daily dose of 2 g. of honey contained no appreciable quantity of vitamin A.

Vitamins B_1 and B_2.

Vitamins B_1 and B_2 were estimated on the principle described by Chick and Roscoe [1927, 1928], whose help and criticism I wish gratefully to acknowledge. The *L* diet described by these authors was employed as the basal diet. The caseinogen used was B.D.H. "fat-free, vitamin-free casein" and not the specially extracted caseinogen used by them. Six groups of rats were used in the tests and were arranged as follows: group 1, basal diet; group 2, basal diet + 2 g. daily of English honey; group 3, basal diet + 2 g. daily of West Indian honey; group 4, basal diet with 0·4 g. autoclaved yeast + 2 g. English honey daily; group 5, basal diet with 0·4 g. autoclaved yeast + 2 g. West Indian honey daily; and group 6, basal diet and 0·4 g. dried yeast daily. The autoclaved yeast was given to supply vitamin B_2, so that in the event of vitamin B_1 being present in the honey the animals receiving this yeast would have shown growth. The animals in all groups received 3–5 drops of cod-liver oil daily as a source of vitamins A and D. Fig. 2 gives the weight curves of the animals in these groups. With the exception of the positive controls in group 6, the animals of which received both vitamins B_1 and B_2 in the dried yeast

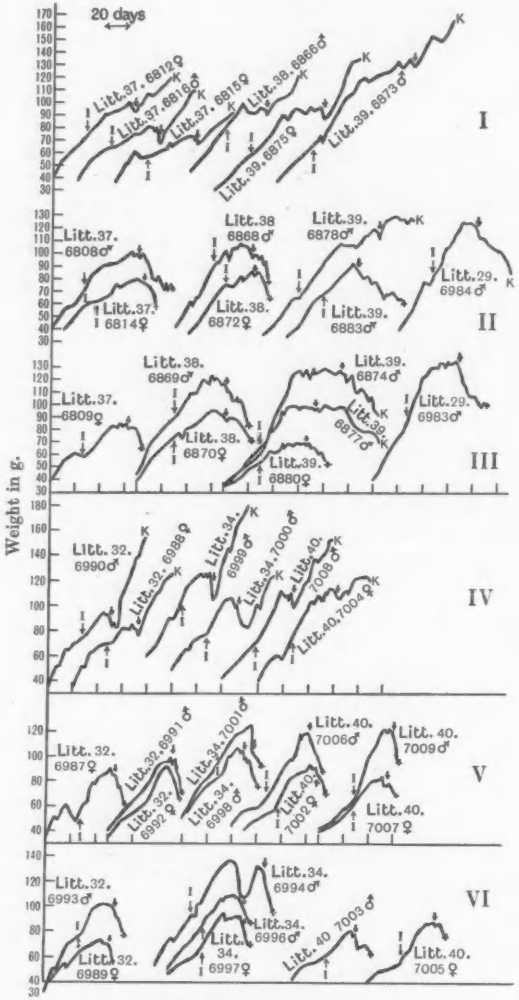

Fig. 1. Test for vitamin A in honey.

I ↓ denotes when irradiated diet first received.

K at end of a graph denotes that animal survived the period of 28 days after giving dose.

↓ denotes when animal began to receive honey.

I Positive controls for West Indian honey.
II West Indian honey.
III Negative controls for West Indian honey.
IV Positive controls for English honey.
V English honey.
VI Negative controls for English honey.

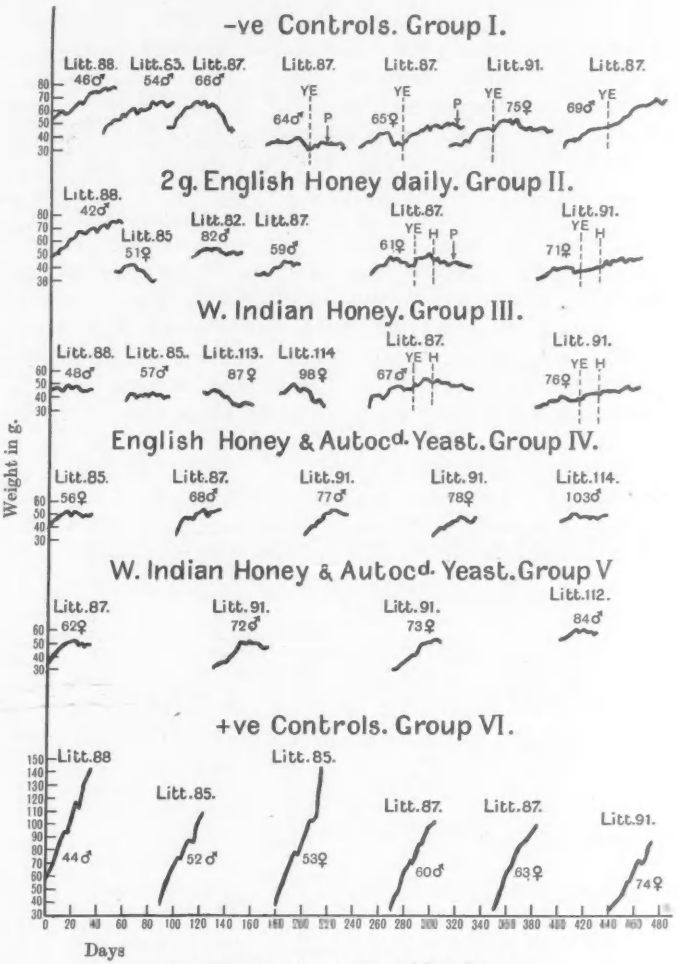

Fig. 2. Test for vitamins B_1 and B_2 in honey.

YE — denotes when Peters's extract was first given.

H — denotes stoppage of daily dose of honey.

P — denotes definite symptoms of pellagra.

there was little growth. Rats 61, 64, 65, 67, 69, 71, 75 and 76 were given by Miss Chick and Miss Roscoe after the 34th day a daily dose of 0·1 cc. of Peters's antineuritic extract (0·1 cc. daily equal to 0·7 g. yeast) to supply vitamin B_1, and, as will be seen from the weight curves of these animals, no resumption in growth followed this treatment. It may, therefore, be safely assumed that both samples of honey were deficient in vitamins B_1 and B_2.

Vitamin C.

Guinea-pigs subsisting on a scorbutic diet of oats, bran, barley meal, middlings and fish meal, which was offered *ad lib.*, and 40–60 cc. of autoclaved milk, were used in this test. The honey was diluted with about one-third of its weight of water and was administered daily to the animal from the commencement of the experiment. The initial daily dose was 3 g. and it was gradually increased. Certain difficulties were encountered with these tests as the guinea-pigs showed a tendency to succumb to an intestinal disturbance in the early stages of the tests and conclusions had to be drawn from such animals as survived long enough for the development of scurvy. The results are summarised in Table I. It is evident from the figures that although death was somewhat accelerated in certain cases by intestinal disturbance, there was not sufficient antiscorbutic potency in the doses given even to delay the onset of the disease.

Table I. *Test for vitamin C in honey.*

Variety of honey	Ref. no. of guinea-pig	Initial wt. g.	Maximum wt. g.	Final wt. g.	No. of days alive	Average daily consumption of honey g.	Day of 1st appearance of scurvy	P.-M. remarks
West Indian	399	315	370	280	27	4·4	15	Died of scurvy
	405	260	295	255	19	5·3	17	Degree of scurvy was such as to indicate that dose was completely negative, though death was due in part to an intestinal disturbance
	406	255	300	220	32	5·1	17	Died of scurvy
	407	260	320	210	28	5·2	17	Died of scurvy
	408	285	287	205	27	5·1	14	Died of scurvy
	409	260	280	250	22	5·4	16	Same condition as no. 405
English comb	410	265	300	240	23	5·3	15	Died of scurvy
	412	285	320	255	22	5·2	14	Scurvy and intestinal disturbance
	413	260	305	200	33	5·1	14	Died of scurvy
	414	260	315	225	29	5·2	14	Died of scurvy
	416	260	260	205	23	4·7	15	Scurvy and intestinal disturbance
	417	260	260	190	25	4·7	18	Died of scurvy
	418	285	290	235	24	5·7	17	Died of scurvy
	419	285	330	255	23	5·9	15	Died of scurvy

Vitamin D.

This vitamin was determined by the Chick, Korenchevsky and Roscoe [1926] method which is based on the comparison of the ratios of the ash to the organic material, other than those substances extractable by hot alcohol and ether, (A/R ratios) of the bones of young rats which have subsisted for about 4 weeks on McCollum's 3143 rickets-producing diet low in phosphorus.

Table II. *Test for vitamin D in honey.*

Rat no.	Initial wt. in g.	Final wt. in g.	Weight of bone g.	% water	% fat	% organic residue	% ash	*A/R*	Average *A/R*
Positive controls for West Indian honey.									
1 ♀	40	59	0·8407	54·15	4·85	21·54	19·45	0·903	
2 ♂	40	55	0·8139	55·68	6·12	21·26	16·94	0·797	
8 ♂	41	67	1·0236	51·56	6·96	22·70	18·77	0·827	
11 ♂	40	62	0·9800	55·82	6·76	20·79	16·64	0·801	
12 ♂	41	52	0·8590	55·56	5·93	20·96	17·54	0·837	
17 ♀	38	56	0·8048	53·88	4·87	22·23	19·02	0·856	
18 ♂	40	57	0·9040	54·78	6·08	21·79	17·34	0·796	
22 ♂	43	70	0·9396	54·42	6·66	21·09	17·82	0·845	0·833
2 g. *West Indian honey daily.*									
3 ♀	40	66	0·8829	62·04	4·06	22·64	11·28	0·499	
4 ♂	41	75	1·0293	60·33	5·53	22·04	12·12	0·550	
7 ♂	41	72	1·0670	61·33	4·02	23·41	11·23	0·480	
9 ♀	37	56	0·8331	59·99	5·58	21·80	12·63	0·579	
10 ♂	39	67	0·9225	62·93	4·61	21·55	10·91	0·507	
19 ♀	43	70	0·8752	59·44	3·27	22·40	14·89	0·665	
20 ♂	42	74	1·0725	61·08	4·43	21·44	13·04	0·608	
21 ♀	43	75	0·9456	62·34	3·24	23·46	10·96	0·467	0·544
Negative controls for West Indian honey.									
5 ♀	41	72	0·9447	63·67	3·12	22·89	10·31	0·451	
6 ♂	41	76	0·9438	63·44	3·78	22·68	10·08	0·445	
13 ♂	41	65	0·9187	63·53	2·78	22·52	11·18	0·497	
14 ♂	40	63	0·9475	62·70	4·98	22·07	10·27	0·465	
15 ♀	43	77	0·9305	61·64	3·63	22·26	12·47	0·560	
16 ♂	42	68	0·9556	63·83	3·33	22·29	10·54	0·473	
23 ♂	44	76	0·9610	59·60	5·00	21·61	13·79	0·638	0·504
Positive controls for English comb honey.									
24 ♀	45	59	0·7873	55·49	7·06	21·42	16·02	0·747	
25 ♂	45	57	0·8233	50·79	9·97	20·91	18·31	0·876	
26 ♀	45	57	0·8309	52·36	8·55	21·37	17·72	0·827	
36 ♂	42	69	0·7506	53·26	6·32	21·54	18·87	0·878	
37 ♀	39	60	0·8693	54·80	7·82	21·20	16·18	0·763	
38 ♂	40	67	0·9292	53·30	8·50	21·13	17·07	0·807	0·812
2 g. *English comb honey daily.*									
27 ♂	43	71	0·9546	63·78	5·97	20·84	9·42	0·452	
28 ♀	43	68	0·8510	63·62	3·73	22·84	9·81	0·429	
29 ♂	50	78	0·9449	62·14	4·89	22·84	10·06	0·440	
39 ♀	35	52	0·6489	63·33	4·41	23·93	8·32	0·347	
40 ♂	39	58	0·7509	63·98	4·07	23·03	8·92	0·386	
41 ♀	41	58	0·7524	64·49	3·07	24·64	7·80	0·317	0·395
Negative controls for English comb honey.									
30 ♀	43	70	0·8759	62·66	4·69	22·32	10·35	0·464	
31 ♂	45	79	0·9889	62·62	4·49	22·62	10·26	0·454	
32 ♂	48	75	0·9362	61·94	4·43	22·05	11·57	0·525	
33 ♀	39	54	0·7329	62·91	4·06	24·51	8·52	0·348	
34 ♂	41	63	0·8126	65·88	3·62	22·92	7·60	0·333	
35 ♀	38	59	0·7987	64·91	3·42	23·58	8·09	0·343	0·411

The animals received a daily dose of 2 g. of each of the honeys prepared as above. With each honey a group of negative control animals on the basal diet only and a positive group which received 3 drops of cod-liver oil in addition were employed. As will be seen from Table II the differences between the A/R ratio of the rats receiving the honeys and those of the corresponding negative control animals are of an order which falls within the limits of accuracy of the method. It may be safely assumed that both the honeys were inactive in the doses employed in the test.

Conclusion.

Tests carried out on a fresh English sample of comb honey and on a West Indian honey show that both samples are deficient in vitamins A, B_1, B_2, C and D. These results are in consonance with those obtained by other workers, which show that honey is not a source for these vitamins and that this deficiency is not due to deterioration consequent on treatment or storage.

My thanks are due to Dr S. S. Zilva, under whose direction the investigation was carried out, for help and criticism.

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X. THE RELATION OF THE GROWTH OF CERTAIN MICRO-ORGANISMS TO THE COMPOSITION OF THE MEDIUM.

IV. THE ADDITION OF MANNITOL.

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In a paper by Orr-Ewing and Reader [1928], a technique was described for using the growth-promoting power of yeast concentrates upon *Streptothrix corallinus* as a test for the antineuritic vitamin. In this technique the yeast concentrate under test was put up in three dilutions, viz. 1/20, 1/400, 1/8000 in equal volumes of medium (20 cc.), and the resulting streptothrix growth compared with that in a series of standard flasks containing similar dilutions and intermediate dilutions of a concentrate of known potency. The result was expressed in terms of pigeon doses. When testing the purer antineuritic concentrates agreement with the corresponding standards was obtained in all three dilutions, *e.g.* 80, 70, and 75 doses in a given test sample. However, certain "anomalous results" appeared with the less pure concentrates; the tests came out thus, 500, 70, 75; *i.e.* there appeared to be relatively more growth-promoting activity in the higher concentrations of vitamin than in the lower. In view of this discrepancy, further work was undertaken to elucidate the phenomenon.

It has often been noted that the upper limit of concentration necessary for maximum growth is reached when approximately 1/80 of a pigeon-day-dose has been added to 20 cc. of the medium. Further addition of glucose or of vitamin concentrate at this point does not induce further growth. Now the anomalous result always occurred when the yeast concentrate was comparatively impure, and this suggested that the abnormally high results with the larger additions of vitamin were due to some added source of food for the organism. A few preliminary experiments done in 1924 had seemed to suggest that some specific carbohydrate or polyhydric alcohol might give greater growth in a given time at a given temperature than that obtained with glucose. Further, from earlier published work [Reader, 1927] it was known that "the following substances may replace glucose as a source of carbon in the medium, without loss of efficiency: glycerol, mannitol, arabinose, lactate, citrate, pyruvate, and glycogen." As these earlier experiments were carried out in a medium of pure salts + carbon compound, it was now decided to test their effect as additional substances to the seemingly complete medium.

Experimental.

The technique differs from that described in Part III [Reader, 1928] in one particular only, that of method of estimation. Since all the experiments in this section are concerned with the requirements for maximal growth and not with minimal dilutions for growth, it was possible to estimate the amount of growth by direct weighing. The contents of three small similar conical flasks (20 cc. fluid) were filtered through the same small, hard, dried and weighed filter-paper. The whole was then dried in a $CaCl_2$ desiccator to constant weight. Since the variation in twenty similar papers was not greater than $\pm$ 5 %, this method was considered to be of sufficient accuracy. Growth was estimated at the end of 6 days, unless otherwise stated. Conditions of temperature are quoted at the beginning of each experiment.

Exp. 1. The amounts of synthetic medium, glucose, and torulin (vitamin) extract were kept constant throughout seven sets of three flasks, while 0·5 % of the various compounds under test was added severally to each of the sets (Table I).

Table I.

T = 1/100 unit dose of torulin in 20 cc. fluid.
Temp. 31° for 2 days followed by 15° for 4 days.

Set	Medium	Total weight from three flasks g.
I	Salt-sugar medium + T	0·025
II	" " + T	0·025
III	" " + T + arabinose	0·024
IV	" " + T + glycogen	0·021
V	" " + T + glycerol	0·041
VI	" " + T + citrate	0·065
VII	" " + T + mannitol	0·144

It will be noted that the six-fold increase of growth with mannitol is of the order of the divergence of the "anomalous results" from the normal. Consequently Sets I and VII were repeated, dilutions being made as in the test cases described in the paper by Orr-Ewing and Reader [1928], in each case the mannitol being diluted with the torulin.

When torulin and mannitol were diluted 400 times, *i.e.* as in the third flask of the test, growth was equivalent to that with torulin without mannitol, hence a true case of "anomalous result."

Exp. 2. The growth obtained in Set VII of exp. 1 was so prolific that it was decided to extract the dried organisms with ether to find out whether the increased weight was due to increased fat production rather than to an increase in the number of organisms (see Table II).

Table II.

	Weight of organism g.	% fat-soluble material
I	Growth without mannitol 0·045	6·0
II	Growth with mannitol 0·144	6·1
III	" 0·142	6·0

From these figures it was concluded that mannitol had not increased the fat production.

Exp. 3. The mannitol used in the above experiments was as supplied by Baird and Tatlock and was supposed to be pure. However, considering the earlier experience with traces of accessory factor present as impurity in glucose [Reader, 1928] an attempt was now made to purify the mannitol from any such factor. In the first place the mannitol was tested before and after extraction with charcoal in neutral solution, but this was found to have no effect, the respective growths being 0·135, 0·133 and 0·132 for the original mannitol and after one and two treatments with charcoal.

While developing a technique for the extraction of the antineuritic vitamin Kinnersley and Peters [1925] found that control of hydrogen ion concentration was an important factor in the removal of such accessory factors by charcoal adsorption. A more exhaustive attempt at removal on charcoal at various hydrogen ion concentrations was now undertaken, but again without success (Table III).

Table III.

T = 1/100 unit dose of torulin in 20 cc. fluid.
Temp. 31° for 2 days followed by 15° for 4 days.

Set	Medium	Weight of growth g.
I	Salt-sugar medium $+T$	0·033
II	" " $+T+M$ (crude, 0·1 g. in 20 cc.)	0·140
III	" " $+T+M$ (purified charcoal at $p_H=2$)	0·137
IV	" " $+T+M$ (purified charcoal at $p_H=5$)	0·138
V	" " $+T+M$ (purified charcoal at $p_H=7$)	0·135
VI	" " $+T+M$ (purified charcoal at $p_H=9$)	0·139
VII	" " $+T+M$ (hydrolysed dilute HCl for 30 mins.)	0·127
VIII	" " $+T+M$ (hydrolysed dilute NaOH for 30 mins.)	0·121

As the results show, no change was produced by these various modes of treatment. Additional proof that the growth-promoting property is due to the presence of the mannitol itself, and not to an impurity, was obtained by reducing a sample of mannose which had been previously tested and found to have no enhancing effect on the growth (Table IV). The reduction was carried out according to the directions of Fischer [1890], using Na amalgam in alkaline solution with rapid mechanical stirring for 12 hours.

Table IV.

Set	Medium	Growth g
I	Salt-sugar medium $+T$	0·033
II	" " $+T+$mannose (0·5 %)	0·028
III	" " $+T+$mannitol (0·5 %) (from mannose)	0·090

It was now decided to test other available polyhydric alcohols. Similar experiments were designed with dulcitol, sorbitol, glycerol and inositol (Table V). From exp. 5, Table V it may be seen that the effect with none of them was of the order of that obtained with mannitol, *i.e.* the enhancement with mannitol appears to be a specific one for this organism.

Table V.

Set	Medium	Weight of growth g.
I	Salt-sugar medium + T	0·033
II	„ „ + T + mannitol	0·140
III	„ „ + T + dulcitol	0·065
IV	„ „ + T + sorbitol	0·045
V	„ „ + T + inositol	0·057
VI	„ „ + T + glycerol	0·058

With regard to the phenomenon of the optimum concentration of the growth factor referred to in the introduction (p. 61) it now seemed possible that, in the presence of mannitol, any excess of growth-promoting factor supplied might be used up. In the following experiments the amounts of mannitol and glucose were kept constant throughout, while the torulin concentrate was varied from 1/20 to 8/20 day-dose per 20 cc. The results are shown in Table VI. From these, it may be concluded that there is a definite upper limit of torulin concentrate for maximum growth even in the presence of mannitol, but that that upper limit is much greater than with glucose alone, as is also the maximum growth obtained. It is easily seen from the curves for the four experiments cited that in each case the addition of mannitol has led to an abnormally high value for the maximum growth. Therefore, with the highest concentration of torulin each case is a true "anomalous result."

Table VI.

(All flasks contain 20 cc. inorganic salt-sugar medium.)

G = 0·5 % glucose. M = 0·5 % mannitol. T = 1/20 unit dose torulin concentrate.

Exp. 6. Temperature 3 days at 33° then 6 days at 16–20°.
Exp. 7. „ 3 „ 33° „ 6 „ 20–25°
Exp. 8. „ 6 „ 33°.
Exp. 9. „ 6 „ 33° „ 3 „ 16–20°.

	Medium	g. growth			
		Exp. 6	Exp. 7	Exp. 8	Exp. 9
1.	$G+T$	0·056	0·060	0·057	0·061
2.	$G+4T$	0·056	0·061	0·050	0·065
3.	$G+8T$	0·056	0·060	0·059	0·064
4.	$G+M+T$	0·073	—	0·138	0·090
5.	$G+M+2T$	0·090	0·186	0·151	0·113
6.	$G+M+4T$	0·084	0·187	0·161	0·143
7.	$G+M+8T$	0·111	0·180	0·154	0·171

The variations in the results ($G + M + xT$) are due to variation in the average room temperature during the time after the cultures had been removed from the incubator. This was very puzzling at first, until it was realised that not only the torulin efficiency, but also the temperature coefficient had been appreciably altered by the addition of mannitol. Thus, in exp. 6 the temperature on the latter days varied from 16° to 20° and in exp. 7 from 20° to 25°. The difference between the results is greater than in exps. 8 and 9 where the change from the incubator temperature to that of the room occurred at a later stage in the life cycle of the organism [see Reader, 1926].

Much further work is needed upon this point, more especially as the issue

is complicated by the fact that the organism under observation is a *Streptothrix*, that is, it is continually segmenting, and the rate of the segmentation is also varied by slight changes in temperature.

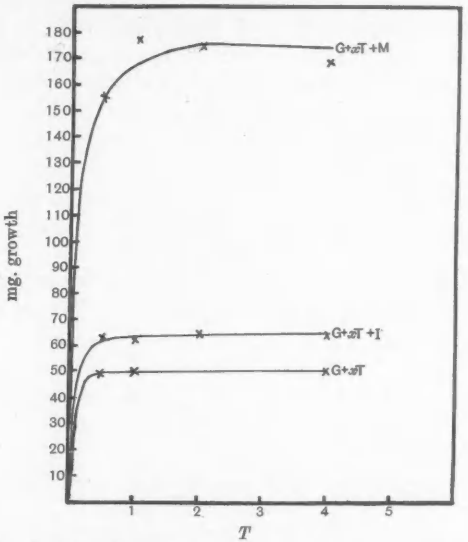

Fig. 1. Weight of organisms in 14 days, *i.e.* after growth is complete.
(M = 0·5 % mannitol; I = 0·5 % inositol; G = 0·5 % glucose; T = 1/20 unit dose of torulin.)

Table VII.

Temperature 7 days at 33°, then 7 days at 20°

I = 0·5 % inositol. M = 0·5 % mannitol. T = 1/20 unit dose torulin concentrate.

	Medium	Weight of growth in 14 days g.
1.	$G+T$	0·050
2.	$G+2T$	0·051
3.	$G+M+1/10T$	0·070
4.	$G+10M+\frac{1}{2}T$	0·160
5.	$G+\ M+\frac{1}{2}T$	0·155
6.	$G+1/10M+\frac{1}{2}T$	0·154
7.	$G+10M+\ T$	0·170
8.	$G+\ M+\ T$	0·179
9.	$G+1/10M+\ T$	0·175
10.	$G+10M+2T$	0·169
11.	$G+\ M+2T$	0·175
12.	$G+1/10M+2T$	0·178
13.	$G+10M+4T$	0·174
14.	$G+\ M+4T$	0·165
15.	$G+1/10M+4T$	0·170
16.	$G+\ I+\frac{1}{2}T$	0·064
17.	$G+\ I+\ T$	0·062
18.	$G+\ I+2T$	0·067
19.	$G+\ \frac{1}{2}I+\ T$	0·057
20.	$G+\ I+4T$	0·063

A final, and perhaps more conclusive experiment was carried out (see Fig. 1). In this the weighings were taken at the end of 14 days. The curve for inositol is added for comparison. The results are given in Table VII. As the growth was independent of concentration of mannitol, at least within these limits, only one curve is drawn ($G + xT + M$) (Fig. 1).

Thus it may be seen that even with 1/200 of a unit dose (1/10 T) the growth with mannitol is in excess of the maximum growth with glucose alone, and also that the maximum growth with mannitol is not reached until 1/20 of a unit dose is supplied in 20 cc. of medium. From this table it is obvious that in the presence of mannitol a true "anomalous result" is artificially reproduced.

Discussion.

Whether the results obtained by Orr-Ewing and Reader with crude torulin extracts were due to the presence of mannitol in the watery extracts of yeast has not yet been proved. Indeed it would appear that the prolonged extractions and purification of yeast extract necessary to show the presence of mannitol would be unlikely to meet with success as the work is complicated by the presence of inositol [see Eastcott, 1928] and possibly other polyhydric alcohols of very similar properties.

At this point it is interesting to consider the parallel case reported by Eastcott [1928]. She claims to have isolated "bios I" [see Lucas, 1924] for yeast, and to have identified this substance with inactive inositol, the "phaseomannite" of Vohl [1856]. However the degree of stimulation reported in her experiments is not greater than that recorded in this paper for the action of mannitol on the growth of *S. corallinus*. Thus, from her experiments, it may be seen that in 48 hours, in solutions containing salts, sugar and "bios II," 113 cells of yeast were produced, while with salts, sugar, crude "bios II" and inositol, some 365 cells resulted from the same size of inoculation. This increase is of the same order as that shown in Tables VI and VII of this paper. Certainly I do not consider that the mannitol effect may be explained as due to a true "growth-promoting factor," but rather as a specific source of food-supply and would suggest a similar interpretation of the results of Eastcott.

Summary.

Further work on the cultural requirements of *S. corallinus* is reported.

1. The "anomalous results" obtained by Orr-Ewing and Reader when crude watery extracts of yeast were added to the culture medium, can be artificially reproduced by adding mannitol to the purer extracts.

2. The increased weight of bacteria grown in the presence of mannitol was not due to increased fat production.

3. Within the limits of these experiments, the amount of growth was independent of the concentration of the mannitol.

4. Exhaustive treatment with charcoal at various hydrogen ion concentrations and subsequent recrystallisation failed to remove any growth-promoting factor from the mannitol.

5. A sample of mannitol was prepared from mannose which had previously been shown to have no growth-promoting effect. This mannitol gave an enhancement of growth equal to that obtained with mannitol from natural sources as usually supplied for laboratory purposes.

6. It is not possible to substitute dulcitol, sorbitol, glycerol, or inositol for the mannitol.

It is concluded that the effects obtained are due to either the whole or part of the mannitol molecule acting as a specific source of food-supply rather than as an additive "growth-promoting factor."

My thanks are due to Prof. R. A. Peters for his continued criticism and advice, and to the Medical Research Council for a grant.

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XI. THE DIALURIC ACID-ALLOXAN EQUILIBRIUM.

By GEORGE MAXWELL RICHARDSON (*1851 Exhibition Research Scholar*) and ROBERT KEITH CANNAN.

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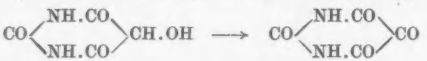
In acid solution, dialuric acid and alloxan may readily be converted into one another. The change is one involving two equivalents of hydrogen:



The addition of one equivalent of a reducing agent to alloxan or of one equivalent of an oxidising agent to dialuric acid leads, on the other hand, to the separation of the sparingly soluble substance, alloxantin. The latter is also formed when solutions of these two reactants are mixed in equivalent proportions, and it has therefore been natural to regard it as a molecular compound comparable to the quinhydrones and meriquinones. It is generally believed that alloxantin is extensively dissociated into its two components in aqueous solution.

Our interest in the above system originated in the demonstration by Biilmann and Lund [1923] that solutions of alloxantin developed constant and reproducible potentials at a platinum electrode in the absence of oxygen. Their observations, which were restricted to solutions of alloxantin in 0·1 and 0·02 *M* sulphuric acid, indicated that the observed potentials (E_h) were related to the hydrogen ion activity by the equation[1]

$$E_h = E_0 - \frac{RT}{F} \ln [H^+],$$

E_0 being a constant with a value of 0·3698 at 18° and of 0·3664 at 25°. Assuming that alloxantin dissociated into equivalent amounts of dialuric acid and alloxan, Biilmann and Lund plausibly attributed the potentials of solutions of alloxantin to the reversible oxidation-reduction system

$$\text{dialuric acid} = \text{alloxan} + 2e.$$

The studies which will be reported in this paper have had as their object the quantitative proof of this assumption, the extension of the electrode data

[1] $[H^+]$ refers throughout to the hydrogen ion activity measured electrometrically. $[a]$, $[b]$ and $[m]$ of the electrode equations are actually molar concentrations, the assumption being made that these are sensibly equal to the activities at the experimental concentrations employed.

to cover the whole significant p_H range, and the examination of the effects upon the electrode behaviour of the system of the formation of alloxantin. It early became apparent that the system was further complicated by the fact that the oxidant was subject to an irreversible change at a rate which was a function of the hydroxyl ion concentration, and it therefore became necessary to relate the kinetics of this change to the electromotive behaviour of the system.

Experimental.

The dialuric acid, alloxan and alloxantin which were employed were prepared from uric acid in the usual way, and their purity established by determinations of nitrogen, base-binding capacity, and (in the cases of dialuric acid and of alloxantin) their iodine-reducing capacities.

In order to comprehend the relations of the equilibrium potentials to $[H^+]$, it was desirable to have independent determinations of those ionisation constants of the reactants which were effective within the experimental p_H range. The acidic constant of dialuric acid was measured with precision by means of a hydrogen electrode titration curve in the usual manner. This titration betrayed no other groups having constants of significant values. The case of alloxan was more difficult. Its ease of hydrogenation renders the hydrogen electrode method inadmissible. Moreover, the degree of ionisation of alloxan determined experimentally appears to change with time. Woods [1906] found that the conductivity of solutions of alloxan increased steadily, and Biilmann and Bentzon [1918] observed that there is also an increase in their $[H^+]$ as determined by colorimetric methods. These effects are, most probably, due to the isomeric change to alloxanic acid which readily takes place under the influence of hydroxyl ions. In an attempt to construct a titration curve by means of colorimetric p_H determinations, we observed this change in p_H in all our mixtures of alloxan and sodium hydroxide. For any given mixture, however, $d\,(p_H)$ is approximately a linear function of the time. It was, therefore, possible to extrapolate to zero time the p_H values of such mixtures (where α varied from 0·09 to 0·58). The section of the titration curve thus constructed led to a quite consistent series of values for the first (acidic) constant of alloxan. It will be shown, later, that the electrode data indicate the presence of a second acidic constant of significant magnitude as well as a very weak basic group. The constant of the latter seems to be too small for direct measurement [cf. Biilmann and Bentzon, 1918] while the demonstration of the former was not attempted because of the instability of alkaline solutions.

The technique employed in the determination of the electrode potentials has already been outlined [Cannan, 1926]. Both dialuric acid and alloxantin proved to be very prone to autoxidation. It was necessary, therefore, to employ all the usual precautions for the total exclusion of oxygen during the preparation and manipulation of solutions of the reactants. Electrode potential titration curves for the titration of dialuric acid (0·002 M) in 0·05 M buffer

solutions [Clark, 1928] with 0·02 *M* potassium ferricyanide or iodine were obtained at several p_H values between 1 and 5. Both platinum and gold-plated electrodes were employed and generally gave concordant readings. The former, however, were frequently sluggish in reaction, whereas the gold-plated electrodes consistently adjusted themselves to changes in the system within two or three minutes. The main part of the curves traced by titrating with iodine and with ferricyanide were identical, but iodine sometimes gave distortions of the initial and terminal portions of the curves, so rendering less precise the determination of end-points. For the detailed analysis of the data, therefore, we have restricted ourselves to the ferricyanide titrations and the gold-plated electrodes. All electrometric observations were made on solutions maintained at 30° in a thermostat.

The titration data for the relation of the electrode potentials to $[H^+]$ were further elaborated by observations of the equilibrium potentials of 0·001 *M* solutions of alloxantin in a series of 0·05 *M* buffer solutions with p_H values intervening between those of the complete titration curves.

On the alkaline side of p_H 5 steady potentials were never attained, either in solutions of alloxantin or in mixtures of alloxan and dialuric acid. Instead, linear drifts towards increasing negative values were consistently observed. It was, therefore, not possible to conduct titrations in this range, but it proved to be possible to obtain consistent values for the equilibrium potentials of alloxantin by extrapolating to zero time the potential-time drifts. Theoretical justification for this procedure will be given later.

The p_H values of all systems studied were determined directly by means of the hydrogen electrode.

Results.

In Table I are assembled the data for a typical titration curve at p_H 1. At constant p_H the electrode equation for the system dialuric acid-alloxan is

$$E_h = E_0' - 0{\cdot}03006 \log \frac{[b]}{[a]}, \qquad \ldots\ldots(1)$$

E_0' being a constant, and $[b]$ and $[a]$ being the respective concentrations of dialuric acid and alloxan. The constancy of E_0' when this equation is applied to the data is satisfactory evidence that the potentials are related to a simple reversible system involving two equivalents of hydrogen.

The first three values for E_0' in Table II have been derived from complete titrations in this way. The remaining values are equilibrium potentials of solutions of alloxantin derived by direct observation or by extrapolation. All these and others are displayed in Fig. 1, which sufficiently demonstrates that the alloxantin potentials agree well with the values of E_0' obtained by titration, and that these are plausibly extended into the alkaline ranges by the values obtained by extrapolation of the time-potential drifts. In other words, the potentials of alloxantin may properly be attributed to the presence in solution of equivalent amounts of dialuric acid and alloxan.

Table I.

Titration of 50 cc. of 0·00175 *M* dialuric acid with 0·02024 *M* potassium ferricyanide in 0·1 *M* HCl. Temperature 30°. p_H 1·043.

Titre	$0{\cdot}03006 \log \frac{[b]}{[a]}$	E_h	E_0'
0·15 cc.	+0·0525	+0·2505	[+0·3030
0·23	0·0468	0·2576	0·3044
0·35	0·0412	0·2620	0·3032]
0·55	0·0350	0·2669	0·3019
0·85	0·0288	0·2730	0·3018
1·15	0·0243	0·2775	0·3018
1·65	0·0187	0·2833	0·3020
2·35	0·0127	0·2888	0·3015
3·15	0·0070	0·2948	0·3018
4·15	+0·0008	0·3011	0·3019
5·15	−0·0054	0·3070	0·3046
6·15	0·0123	0·3138	0·3015
6·95	0·0192	0·3211	0·3019
7·65	0·0279	0·3297	0·3018
8·15	0·0393	0·3415	0·3022
8·35	0·0487	0·3503	0·3016
8·55 (end-point)			
			Mean 0·30185±0·00035

Table II.

Relation of E_0' to p_H. $E_0 = +0{\cdot}3640$.

p_H	E_0' Found	E_0' Calculated	Difference	Method
1·043	+0·3018	+0·3015	0·0003	Titration of 0·002 *M* dialuric acid
2·943	0·1976	0·1979	0·0003	
4·884	0·1320	0·1322	0·0002	
2·127	0·2388	0·2385	0·0003	Equilibrium potentials of 0·001 *M* alloxantin
2·549	0·2143	0·2164	0·0021	
3·153	0·1879	0·1891	0·0012	
3·757	0·1668	0·1675	0·0007	
4·155	0·1549	0·1546	0·0003	
4·565	0·1426	0·1420	0·0006	
4·965	0·1294	0·1297	0·0003	
5·420	0·1155	0·1158	0·0003	
5·885	0·1009	0·1014	0·0005	
6·555	0·0789	0·0790	0·0001	Extrapolated potentials for 0·001 *M* alloxantin
7·027	0·0602	0·0611	0·0009	
7·494	0·036	0·039	0·003	
8·025	+0·011	+0·011	—	
9·007	−0·052	−0·048	0·004	
9·955	0·112	0·112	—	
10·370	0·146	0·144	0·002	
11·055	0·205	0·202	0·003	
11·955	0·274	0·282	0·008	

Table III.

Dissociation constants (uncorrected for activity). Temperature 30°.

	Group	K′	$p_{K'}$	Method
Dialuric acid	1st acidic (K_1')	$1{\cdot}48 \times 10^{-3}$	2·83	Hydrogen electrode E_0' : p_H relation
	2nd ,,	Negligible	>11	
	3rd ,,	,,	>11	,,
Alloxan	1st acidic (K_1')	$6{\cdot}31 \times 10^{-8}$	7·20	Colorimetric E_0' : p_H relation
	2nd ,, (K_2')	$1{\cdot}0 \times 10^{-10}$	10·0	
	Basic	Negligible	<1	,,

Fig. 1 betrays a system more complicated than was expected. The "0·09 slope" exhibited at extreme alkalinity together with the fact that both dialuric acid and alloxan have acid properties suggest that the type of system which best fits the structural relations of the reactants is

$$\left[\begin{matrix}\text{reductant}\\ \text{anion}\end{matrix}\right]^{---} = \left[\begin{matrix}\text{oxidant}\\ \text{ampholyte}\\ \text{anion}\end{matrix}\right]^{+--} + 2e$$

The values to be assigned to the dissociation constants of the various ionisable hydrogen atoms are given in Table III. The strong acid group of dialuric acid must be assigned to the carbon atom carrying the hydroxyl group, and, according to the scheme, this atom must, therefore, assume basic properties on oxidation. Indeed, alloxan may, as has been suggested by Richter [1911], be

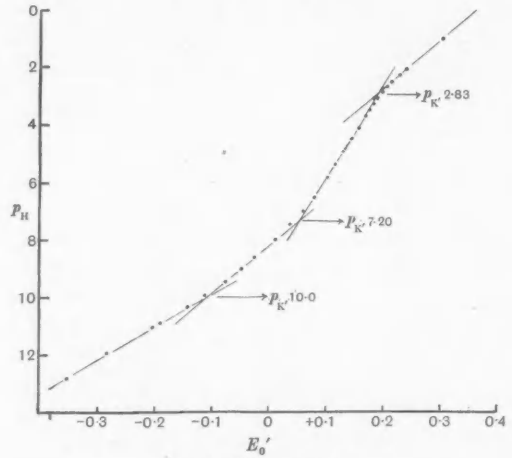
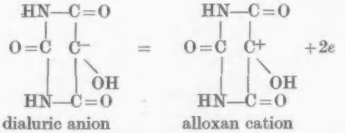


Fig. 1. E_0': p_H curve

an oxonium base, though our allocation of constants would give it only negligible basic properties. The remaining acidic hydrogen atoms of the two reactants may, plausibly, be identified with the hydrogen atoms attached to nitrogen:



With the assumptions of Table III, the rational equation relating E_0' to p_H may be simplified to

$$E_0' = E_0 + 0{\cdot}03006 \log \frac{[H^+]^3[K_r' + H^+]}{[K_1' + H^+][K_2' + H^+]}. \qquad \text{.....(2)}$$

Adopting a value of + 0·3640 for the constant E_0 there may be calculated the theoretical values of E_0' found in Table II. In acid solutions, where the

experimental values are true equilibrium potentials, the agreement is within the experimental error. In neutral and alkaline solutions, where the quoted values are extrapolated from the time course of a drifting potential, greater latitude must be allowed. When it is pointed out that the significant range of potential drift is less than 0·05 v. and that the observed rates rose to values as great as 0·02 v. per minute, it is apparent that precise extrapolation is quite impossible.

It is well known that the initial equilibrium of an electrode with an active system is frequently much slower than subsequent adjustments to changes in that system. This was so in the present case. The observations on solutions of alloxantin in which the potential drift was rapid were, therefore, made by adding 4 cc. of 0·01 *M* alloxantin to 30 cc. of a deaerated buffer of acid p_H, and then, after about fifteen minutes, adding the necessary amount (about 10 cc.) of sodium hydroxide to bring the solution to the desired p_H. The exact p_H value of the solution was determined by the hydrogen electrode at the conclusion of the experiment. The drift of potential was measured from the time of addition of alkali. Using this method, it was possible by observations every half minute to establish, fairly definitely, the linear drift even in the cases where the change was most rapid. There can be little doubt that the data for the more alkaline solutions do establish—in spite of their crudeness —the reality of the two inflexions in the $E_0' : p_H$ curve at p_H 7·2 and 10 respectively (Fig. 1).

The value we have found for E_0 at 30° is quite consistent with those obtained by Biilmann and Lund at 25° and at 18°. The three values may be accurately combined in the expression

$$E_0 = 0{\cdot}3784 - 0{\cdot}00048\,t.$$

The degree of dissociation of alloxantin.

In Table I the tacit assumption was made that the active mass of dialuric acid at each stage in the titration was equal to the fraction of the initial concentration which remained unoxidised. The active mass of alloxan was, likewise, identified with the fraction of the initial concentration of dialuric acid which had suffered oxidation. If, however, significant amounts of the two reactants associate to form alloxantin then these assumptions become unsound. Under such conditions it may be shown that the correct equation for a titration at constant p_H is

$$E_h = E_0' - \frac{RT}{2F} \ln \frac{[S_r] - [m]}{[S_0] - [m]}, \qquad \text{.....(3)}$$

where $[S_r]$ and $[S_o]$ are the concentrations of total reductant and oxidant respectively, and $[m]$ is the concentration of alloxantin. The latter is defined by

$$K = \frac{[m]}{[a]\,[b]}. \qquad \text{.....(4)}$$

Now K has been determined by Biilmann and Bentzon from studies upon the solubility of alloxantin in the presence of varying concentrations of added

alloxan. They arrived at a mean value of 39. This would indicate that the degree of association in 0·001 *M* solution would not be sufficient to give values of $[m]$ significant for equation (3). That we were able to ignore this quantity in Table I agrees with this conclusion. At higher concentrations of reactants, however, the degree of association should lead to a distinct distortion of the normal curve. This we found to be the case in the titration of 0·01 *M* dialuric acid at p_H 1 (Table IV). From the titres may be calculated $[S_r]$ and $[S_0]$ in the usual way and thence equation (3) may be solved for $[m]$. Finally K may be calculated from (4). In view of the fact that the method is not well adapted to the accurate measurement of such an association constant, the values derived in Table IV must be held to be in satisfactory agreement.

Table IV.

Titration of 0·00897 *M* dialuric acid with 0·0508 *M* potassium ferricyanide

$p_H = 1·080$. $E_0' = +0·2990$.

Titre	E_h	$S_0 \times 10^6$	$S_r \times 10^6$	$m \times 10^6$	K
0·41	+0·2572	413·5	8483	71·8	25
0·71	0·2655	711·6	8128	94·5	19
1·01	0·2709	1006	7789	114·7	17
1·51	0·2773	1490	7214	150·0	16
2·21	0·2836	2151	6434	249·5	21
3·01	0·2899	2886	5569	223·3	16
4·01	0·2964	3773	4527	345·5	24
5·01	0·3030	4629	3520	432·0	32
5·51	0·3061	5046	3031	243·2	18
6·01	0·3095	5454	2549	199·2	16
6·81	0·3159	6091	1799	179·0	19
7·51	0·3231	6633	1162	136·6	21
8·01	0·3306	7016	712	97·0	23
8·31	0·3375	7241	445	69·7	26
8·82 (end-point)					Mean 21

Table V.

Velocity of decomposition of alloxan. Temperature 30°.

p_H	Change of E_h per min.	$k = \frac{1}{t} \ln \frac{a}{a-x}$
4·97	−0·000034	0·0026
5·31	−0·00010	0·0077
5·74	−0·00016	0·012
6·00	−0·00037	0·028
6·01	−0·00080	0·061
6·56	−0·0027	0·21
7·03	−0·012	0·92
7·49	−0·018	1·4
7·99	−0·020	1·5
8·03	−0·020	1·5
8·80	−0·016	1·2
9·00	−0·020	1·5
9·01	−0·020	1·5
9·79	−0·016	1·2
9·96	−0·014	1·1
10·37	−0·018	1·4
11·06	−0·016	1·2

Our mean value will be seen to be about half that of Biilmann and Bentzon. Since the p_H of the saturated solutions with which these authors worked would be about 2·4, the discrepancy is much too small to be due to a primary

effect of hydrogen ion activity. More plausibly it may be attributed to the difference in temperature, and in ionic strength, of the solutions in the two studies. We may conclude as a first approximation that K is independent of $[H^+]$ in this range.

The rate of isomeric change of alloxan.

There is little doubt that the drifts of potential of solutions of alloxantin towards more negative values are due to a slow loss of active oxidant. Since they are accelerated by hydroxyl ions and parallel the increases in acidity and conductivity of solutions of alloxan, it is most probable that they are due to the isomeric change of the latter to alloxanic acid. Assuming, therefore, the change to be monomolecular, we have for a buffered solution of alloxantin

$$kt = \ln \frac{a}{a-x},$$

where a is the initial concentration of alloxan and $a - x$ the concentration at time t. At this time, also,

$$E_0' - E_h = \frac{RT}{2F} \ln \frac{a}{a-x}.$$

Hence

$$E_0' - E_h = \frac{RT}{2F} kt. \qquad \text{.....(5)}$$

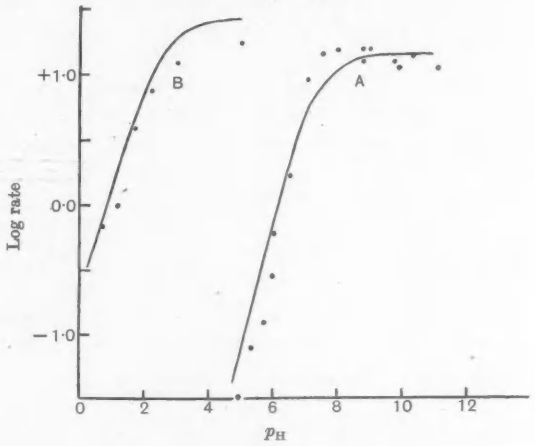


Fig. 2.

A. Isomerism of alloxan (monomol. velocity constant).
B. % autoxidation of dialuric acid per min.

The increase in negative potential should, therefore, be a linear function of the time. As has been already indicated, this agrees with observation. Equation (5) has been employed for the calculation of k from the electrode data at various $[H^+]$. In solutions of p_H greater than 7 the half-life period of alloxan at 30° appears to be only about one minute. The determination of k can, therefore, be only approximate. Nevertheless, as Table V and Fig. 2 indicate,

the velocity appears to be a function of the first acidic constant of alloxan. The curve in the figure represents the theoretical relation of log k to p_H were the velocity a function of the degree of dissociation of alloxan. Beyond p_H 10—coinciding with the intervention of the second (hypothetical) acidic constant—the potential drift falls away from a linear relation although the initial velocities remain of the same order of magnitude.

It may be added that the rates of change in p_H of mixtures of alloxan and sodium hydroxide give general confirmation of the velocities of change indicated above.

The autoxidation of dialuric acid.

To assist in defining the precautions necessary for the electrode studies, a few observations were made upon the rates of autoxidation of dialuric acid in buffered solutions. Between p_H 5 and 2 the rates were very great but fell away rapidly below this.

It was found that when the reaction was conducted by blowing oxygen through a solution of dialuric acid the velocity was greatly affected by the degree of agitation. Consequently, the following method was adopted. Solutions of 0·004 *M* dialuric acid (oxygen-free) were mixed in a closed chamber with five volumes of oxygenated buffer at zero time. Samples were removed at intervals, added to sufficient hydrochloric acid to depress autoxidation, and titrated in the absence of oxygen with 0·005 *M* iodine.

The rate of autoxidation was found to be roughly linear, with a tendency to fall away in the later stages. This was probably due to a significant diminution of the partial pressure of oxygen. The results were not closely reproducible, there being, apparently, uncontrolled catalytic effects. The latter did not however appear to be of the heavy metal type, since the addition of hydrocyanic acid was without notable effect. In Fig. 2 are plotted logarithms of the rates (percentage oxidation per minute) at various p_H values. The comparison curve gives the relation which would be expected were the velocity a direct function of the degree of dissociation of the dialuric acid.

SUMMARY.

1. The equilibrium potentials of the reversible oxidation-reduction system dialuric acid-alloxan have been determined for the p_H range 1–6 and related to the equilibrium potentials of alloxantin observed by Biilmann and Lund.

2. The association constant of alloxantin has been derived from the electrode data.

3. Equilibrium potentials are not obtained in solutions of p_H above 6. This is probably due to the slow isomeric change of alloxan to alloxanic acid. Upon this assumption it has been possible to derive approximate equilibrium potentials for the range p_H 6–12.

4. The first acidic constants of dialuric acid and of alloxan have been determined. A second acidic constant of alloxan is inferred from the electrode behaviour of the system and a value for it derived.

5. Observations have been made on the relation to p_H of the rates of isomeric change of alloxan and of autoxidation of dialuric acid.

One of us (R. K. C.) is indebted to the Medical Research Council for a grant from which the expenses of this work have been defrayed.

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XII. NOTE ON THE DETERMINATION OF TRYPTOPHAN BY MEANS OF *p*-DIMETHYL-AMINOBENZALDEHYDE.

By WILLIAM JOHN BOYD.

(*Received November 19th, 1928.*)

Tryptophan reacts with aldehydes in the presence of acids to give coloured solutions varying in colour according to the aldehyde used.

It is well known that in some cases the reaction requires the presence of an oxidising agent. Thus the colour reaction of formaldehyde with proteins in presence of sulphuric acid is promoted by the addition of a trace of ferric sulphate. Oxidising agents in general have a similar effect on the reaction of tryptophan with benzaldehyde and vanillin respectively, and it appears that this applies generally. Dakin [1906], however, states that the glyoxylic acid reaction (Adamkiewicz test) of tryptophan in presence of sulphuric acid does not require an oxidising agent, but this is true in measure of the reaction with other aldehydes. It can be shown that the presence of reducing substances such as hydrogen sulphide or formaldehyde retards the development of the glyoxylic acid coloration as well as that with other aldehydes.

In carrying out some work on the tryptophan content of fish-muscle protein by means of the method of May and Rose [1922] certain facts came to light which are of considerable importance in the application of this test since large errors may arise if they are ignored.

The May and Rose method is carried out as follows: 0·1 g. protein is added to a mixture of 50 cc. concentrated hydrochloric acid, 50 cc. water and 1 cc. of a 5 % solution of *p*-dimethylaminobenzaldehyde in 10 % sulphuric acid. The mixture is incubated at 36° for 24 hours and then allowed to stand for 24 hours or longer at room temperature. When tryptophan is present a blue coloured solution is obtained. The colour is compared by means of a colorimeter with the colour given by caseinogen under the same conditions, and the tryptophan content is calculated on the assumption that caseinogen contains 1·5 % of tryptophan. Jones, Gersdorff and Moeller [1924] have pointed out that different proteins are hydrolysed under these conditions at different rates and that a longer time must be given for development of the colour.

Holmes and Greenbank [1923] have studied the influence of temperature on the reaction of tryptophan, free and combined in proteins, with *p*-dimethylaminobenzaldehyde. They found that the development of the colour is much more rapid and complete at 37° than at room temperature and also that fading goes on concurrently with colour production. The colour is very stable at

room temperature but fades rapidly at 42°. They chose 37° as a suitable temperature and kept the mixture till a maximum intensity of coloration was obtained. This method gave in their hands good results for solutions of pure tryptophan but they do not state what standard was used. The conditions with proteins are very different since hydrolysis, colour development, and colour fading are all going on at once. For this reason a standard solution of pure tryptophan is not permissible in determining the tryptophan content of proteins if a high temperature is used.

Holmes and Greenbank[8] have also found difficulty in the varying rate of hydrolysis of different proteins and favour the use of enzyme digests.

Some experiments were carried out with a view to explaining large variations in colour intensity obtained using equal volumes of the same trypsin digest of caseinogen or equal weights of the same sample of dry caseinogen. The relative intensities were sometimes as 7 : 10 and it was also found that the order might be reversed, that which at first was less intense becoming the stronger. These variations were traced to accidental differences in the illumination of the two tests. Also, sometimes delay occurred in the development of the colour in solutions of other proteins which caused it to be assumed that only a trace of tryptophan was present, an assumption subsequently disproved by development of considerable coloration. This was traced to the presence of small quantities of reducing substances. It was shown that development of the colour in solutions of pure tryptophan is retarded by (1) the presence of hydrogen sulphide or formaldehyde, (2) a layer of toluene over the reacting mixture, (3) absence of light. It was also found that addition of traces of an oxidising agent brings about rapid development of the colour at room temperature, and that exposure to bright light has a similar effect. The presence of pure isinglass does not hinder the development of colour, it is neither hindered by bubbling carbon dioxide through the reacting mixture in daylight, nor hastened by bubbling oxygen through the reacting mixture.

The following factors will now be considered in turn: (1) effect of reducing substances; (2) effect of oxidising agents; (3) effect of light.

Effect of reducing substances.

Herter [1905] has shown that the colour reaction of urine with *p*-dimethylaminobenzaldehyde (due to indole derivatives) is prevented by the presence of formaldehyde. Acree [1906] and Dakin [1906] also mention that excess of formaldehyde prevents the development of the coloration given by milk with formaldehyde in presence of sulphuric acid. In the course of work on cod-muscle protein it was found that, if this purified protein is soaked in a dilute solution of formaldehyde and then thoroughly washed with cold water, the Adamkiewicz reaction with glyoxylic acid in presence of sulphuric acid and the *p*-dimethylaminobenzaldehyde reaction in presence of hydrochloric acid become faint. The material appears to recover the power of giving positive tests with these reagents on prolonged storage.

These observations, together with the experiment with hydrogen sulphide already mentioned, show that the presence of reducing substances is a disturbing factor in the determination of tryptophan by the method of May and Rose and similar methods using other aldehydes. It follows too that excess of the aldehyde reagent will hinder the development of the colour and may cause colorimetric readings at a given time to be not truly proportional to the tryptophan present. In this connection it may be noted that Holmes and Greenbank found little difference in the results obtained, using 1 mol. of reagent for every mol. of tryptophan and using 10 mols. of reagent for every mol. of tryptophan, but they obtained the best results with 2 mols. It is evident that if only 1 mol. is present the aldehyde reagent will be gradually oxidised so that by the time all the tryptophan has been liberated there will not be the equivalent quantity of aldehyde to combine with it. On the other hand, too great an excess of reagent may retard the oxidative development of colour. Probably at the higher temperature, 37°, this is not so evident as at lower temperatures.

Effect of oxidising agents.

The addition of an oxidising agent at the same time as the reagent is added will obviously alter the aldehyde rapidly before it has time to combine with the tryptophan, especially as the latter is only slowly liberated from the protein. This is confirmed in practice when quantities of the order employed by May and Rose are used. The oxidising agent must be added after hydrolysis is completed.

In making qualitative tests in small volumes of liquid the coloration can be produced at once on addition of an oxidising agent. Small pieces of tissue can be quickly stained for tryptophan in this way. In these instances, however, probably only a small fraction of the tryptophan present is transformed.

In order to compare the coloration obtained with and without addition of an oxidising agent, tests were carried out by the method of May and Rose, using 0·1 g. of caseinogen and cod-muscle protein respectively. Duplicate tests were carried out similarly but 3 drops of 0·5 % sodium nitrite solution were added after 3 days and again after 3 days more. The colorimetric readings were made on the following day using a Duboscq colorimeter and a blue glass screen of the correct tint as standard. They are given in Table I.

Table I. *Comparison of the intensity of coloration obtained with and without addition of sodium nitrite solution.*

Substance	Time at room temp. after 1 day at 38°	Without addition of sodium nitrite	Sodium nitrite added
Caseinogen	7 days	22·9 mm.	15·0 mm.
	31 days	17·4 mm.	
Cod-muscle protein	7 days	34·8 mm.	17·6 mm.

The quantity of tryptophan present is in inverse proportion to the colorimetric reading.

From these results it is evident that the development of colour, without addition of sodium nitrite, is so slow that it does not in 31 days attain to the intensity obtained in 8 days on addition of sodium nitrite. Also it appears that if caseinogen is taken as a standard in order to determine the tryptophan content of cod-muscle protein, a higher result is obtained with sodium nitrite than without it.

If caseinogen is assumed to have 2·2 % tryptophan [cf. Jones, Gersdorff and Moeller, 1924] the values for cod-muscle obtained by the two methods are 1·45 % and 1·87 %. As already explained, variations in the intensity of the illumination in the one case may give variable results.

Similarly for edestin a tryptophan content of 3·50 % was found, which is considerably higher than the value obtained by May and Rose [1922] and by Jones, Gersdorff and Moeller [1924] without addition of an oxidising agent, the same tryptophan content of the caseinogen being assumed in each case.

Addition of a trace of nitric acid or of hydrogen peroxide has a similar effect to that of sodium nitrite.

Effect of light.

It has been mentioned already that the coloration produced in the May and Rose test varies according to the degree of illumination of the solution.

The following experiment illustrates the effect of light in promoting the reaction of tryptophan with *p*-dimethylaminobenzaldehyde. 0·1 g. edestin was added to a mixture of 100 cc. 18 % hydrochloric acid with 1 cc. of a 5 % solution of *p*-dimethylaminobenzaldehyde in 10 % sulphuric acid. This was allowed to stand at room temperature (10°) in the dark for 24 hours. A little gelatinous edestin remained undissolved and was removed by means of a glass rod. Equal portions of the clear colourless solution were decanted into three beakers of 5 cm. diameter. One of these was returned to the dark room, one was placed just inside a closed window and the third outside on the sill. The sky was cloudy but fairly bright. Within 1 minute the solution outside was distinctly blue, whilst no trace of blue colour could be detected in the other portions. In 30 minutes the solution outside was deep blue, that inside the window was faintly blue, whereas that in the dark was quite colourless. A drop of hydrogen peroxide rapidly developed colour in the last-mentioned solution. It is probable that the ultra-violet rays are the active agents since the interposition of glass or toluene makes a considerable difference in the rate of colour development.

From these considerations it appears that the best way to avoid the disturbing effects of varying illumination, and of reducing substances, is to add a little sodium nitrite, nitric acid or hydrogen peroxide to the test after hydrolysis is completed. For example 3 drops of 0·5 % sodium nitrite may be added to the test after incubation for 24 hours at 36° and 3 days at room temperature, and again after 3 days more, the colorimetric comparison being made next day.

The addition of sodium nitrite has been adopted by Fürth and Lieben [1920], using formaldehyde instead of *p*-dimethylaminobenzaldehyde. This addition is now shown to be advisable with the latter reagent.

Undoubtedly the ideal procedure would be to carry out the test on an enzyme-digest of the protein if the tints obtained with these digests were not so variable for different proteins. From some tests made on these lines it appears likely that the tryptophan content of cod-muscle is higher than the value given on page 81, and higher than that of caseinogen, but owing to the difficulty mentioned the results are uncertain.

Summary.

Errors can arise in the determination of tryptophan in proteins by the method of May and Rose (1) through unequal illumination of the reacting mixtures, and (2) through the presence of reducing substances such as hydrogen sulphide or aldehydes.

The development of the colour is an oxidation process which goes on slowly in dull light and more rapidly in bright light. It is not nearly complete in a period of 4 weeks in ordinary diffuse daylight in the laboratory. It can conveniently be hastened by adding a trace of an oxygen carrier or oxidising agent after hydrolysis of the protein.

In carrying out the test 3 drops of 0·5 % sodium nitrite solution should be added to the reaction mixture after 24 hours' incubation at 36° and 3 days at room temperature and again after a further 3 days, the colorimetric comparison being made next day or later.

By this modified method higher values for the tryptophan content of cod-muscle protein and edestin are obtained than by the unmodified method of May and Rose.

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XIII. THE ACTION OF INSULIN IN NORMAL YOUNG RABBITS.

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The early publications by the discoverers of insulin gave evidence that under the influence of insulin the glycogen content of the liver increased. Later, however, contradictory results were obtained. Dudley and Marrian [1923] state that, using normal mice, they found that the sugar disappearing from the blood under the action of insulin is not stored in the liver or the muscles as glycogen. Further they found that a dose of insulin big enough to produce convulsions depletes the liver and muscles of glycogen. Collazo, Händel and Rubino [1924], however, investigating the glycogen of liver and muscles in normal guinea-pigs 4 hours after the injection of 7 units of insulin, found a marked increase in both situations. Their animals were starved for 18 hours and received 3 g. of glucose by stomach tube before the injection of the insulin. The degree of rise in glycogen in these experiments both in liver and muscle was about 50 to 70 % above the controls. Cori [1925] found that starving rabbits showed no appreciable change in their liver-glycogen in the first hour of insulin action and that this was true whether the initial glycogen content was high or low and whether the fall in blood-sugar was large or small. In the second hour after insulin the liver-glycogen was found to remain constant or decrease. Similar results were obtained with mice, the average liver-glycogen of 16 injected animals being 39 % lower than the average of 16 controls. On the other hand completely phloridzinised and starving cats, dogs and rabbits were found to deposit liver-glycogen under the influence of insulin. Cori [1924] found an increased glycogen formation as a result of insulin action. Grevenstuk and Laqueur [1925], however, questioned this result. Frank and Hartmann [1926] criticised the findings of Grevenstuk and Laqueur, and Frank, Nothmann and Hartmann [1925] claim to have found an increase in liver-glycogen in fasting rabbits using small doses of insulin. Barbour *et al.* [1927], using a large number of standard rats, examined the changes in blood-sugar and muscle- and liver-glycogen induced by insulin. The effect produced depended on the dose of insulin. Thus, with 1 or 2 units per kg. body weight the liver-glycogen fell during the first 1½ hours and rose slightly after 2 hours: with 3 units per kg. there occurred a steadily progressive fall in the liver-glycogen. The muscle-glycogen of starving animals fell in all cases after insulin. In fed animals,

however, the total fall in liver-glycogen was almost balanced by a rise in the muscles. Cori and Cori [1928], after studying sugar utilisation in normal fasting rats, concluded that, "after insulin, the utilisation of absorbed sugar in the peripheral tissues (mainly muscles) is increased to such an extent that there is almost no sugar available for glycogen deposition in the liver." On this view, therefore, the main action of insulin is to bring about increased oxidation of glycogen in the peripheral tissues. Other experimenters found definite decreases in muscle-glycogen after insulin, for example, Dudley and Marrian [1923], Andova and Wagner [1924], and Kay and Robison [1924]. According to Hetenyi [1925] the "total sugar" of muscles falls after insulin. The work of Dale and co-workers [1926] has demonstrated that deposition of glycogen can be brought about in the eviscerated spinal cat by means of insulin, whereas without insulin perfusion with glucose does not lead to glycogen formation. Best, Hoet and Marks [1926] regard the immediate action of insulin as being a production of a more rapid combustion of sugar with a coincident further synthesis of glycogen.

It is apparent that as far, at any rate, as the liver-glycogen is concerned no final decision has been reached. The interpretation of any given series of results will naturally depend on the view taken of the glycogenic function. The two main views of the glycogenic function are the so-called storage theory and the secretory theory. The distinction between these views is fundamental: the former states that glycogen is deposited in the liver as a passive store which represents the excess of absorbed over utilised carbohydrate, and that the function of the liver is to keep the blood-sugar below certain levels. The latter regards glycogen as a true internal secretion and considers that the liver is constantly manufacturing this substance and liberating it as glucose in order to keep the blood-sugar above hypoglycaemic levels. The evidence for the secretory view is accumulating rapidly and has been fully discussed by Cramer [1928] and experimentally supported by Markowitz [1925].

A further matter which must be constantly kept in mind is the irreversibility of muscle-glycogen. Once glycogen is formed in the muscles it can no longer contribute to the upkeep of the blood-sugar. The experiments of Mann and Magath [1925] show that in hepatectomised dogs adrenaline can no longer bring about a rise in blood-sugar, in spite of the presence of glycogen in the muscles. From the point of view of available carbohydrate which could conceivably be of value in the relief of insulin convulsions it seems clear that muscle-glycogen is valueless. Strangely enough Chaikoff [1925] suggests that the condition for the recovery from convulsions is the presence of muscle-glycogen.

Dudley and Marrian [1923] concluded that muscle-glycogen was almost completely absent when convulsions occurred, but Chaikoff [1925] showed that this was not the case, a finding with which we entirely agree.

Experimental.

The first point which had to be considered was the choice of animals which could provide reliable controls. Barbour *et al.* [1927] state that rabbits are unsuitable because of the marked irregularity in the glycogen content of the tissues, even when every precaution is taken to have the animals fed and otherwise treated alike. This is certainly true of adult rabbits but it is not for very young ones, particularly if they are from the same litter. The values for muscle-glycogen are not as uniform as those for liver-glycogen. It was therefore decided to breed our own rabbits and carry out comparative experiments on members of the same litter. The animals were allowed to grow for periods of about 6 weeks, their diets being bran, oats and abundant green stuff as soon as they became independent of the mother. Occasionally there is some difficulty in keeping them healthy, as they are very susceptible to infections. Our best litters were obtained from primiparae, particularly if the young were all dominants.

The young animals were placed in a cage with no food for 24 hours before experiment. They were then divided into two groups, one being taken for controls. Only the most definitely healthy animals were taken.

Blood-sugar was determined by MacLean's method, heart-blood being generally taken.

Glycogen was determined in liver and muscle in the following manner. The animal was killed by a sharp blow on the back of the head, extended and the thorax and abdomen were rapidly opened. 0·2 cc. blood was taken immediately from the heart and transferred to the sodium sulphate used in the blood-sugar method. The thorax was packed with swabs and the liver quickly removed, the gall bladder detached and the liver weighed. Boiling 60 % potassium hydroxide (1 cc. to 1 g. tissue) was added. This process, from the time of killing the animal until the addition of the potassium hydroxide, did not occupy more than 90 seconds. In general the whole liver was worked up, but in a few cases part of it was taken for the estimation of fat. The liver was never minced but cut into small pieces with sharp scissors.

Meanwhile the posterior extremities were skinned and the gastrocnemii muscles removed, weighed and worked up for glycogen in the usual way. The separation of the muscles is easy in young rabbits because there is very little fat and one obtains what is almost all muscle tissue. The precipitate of glycogen was allowed to settle during 24 hours, filtered, washed twice with 66 % alcohol, twice with absolute alcohol and with absolute ether. The glycogen was dissolved in boiling water which was allowed to filter through into a volumetric flask. An aliquot part was hydrolysed in 1·8 % HCl, until no more glucose was produced. The period was usually 2 hours when the total glucose was not greater than 60 mg. After cooling and neutralising with KOH, the solution was made up to a known volume and filtered through a starch-free filter-paper.

The glucose was estimated by the method of von Issekutz and von Both

[1927] which depends on the reduction of potassium ferricyanide as in the Hagedorn and Jensen blood-sugar method, and the quantities have been so adjusted that amounts of glucose only up to 15 mg. can be estimated. The technique is particularly suitable for accurate glycogen estimations. In general, in this work, the quantities were adjusted so that no estimation involved more than 10 mg. of glucose. Many duplicates were carried out, always with the most satisfactory results.

The insulin used in these experiments was that prepared by Messrs Burroughs and Wellcome. Dilutions were made up in distilled water so that 1 cc. contained 1 clinical unit, and were used only on the day of preparation.

Exp. 1. Six young rabbits, 10 weeks old, from the same litter. Starvation for 24 hours before experiment. Three were used as controls and three were injected subcutaneously with 0·5 clinical unit of insulin. The first of the insulinised animals was killed 1 hour after the injection and the remaining two at the first sign of convulsions. The onset of convulsions in these young animals is always introduced by a period of incoordination followed by flaccidity. These are followed generally by a certain period of recovery, then a typical convulsion. In this experiment the animals were not allowed to go into convulsions but killed at the onset of flaccidity.

Blood for sugar estimation was taken from the heart. The fat content of the liver was also determined in this experiment.

Controls.

No.	Wt. (g.)	Blood-sugar (g. per 100 cc.)	Glycogen (%) Liver	Glycogen (%) Muscle	Liver-glycogen (mg.)	Liver-fatty acid (%)	Time after insulin (hrs.)
1	338	0·077	0·33	0·12	31	3·91	—
2	397	0·162	0·32	0·16	38	8·59	—
3	373	0·120	0·31	0·21	30	7·75	—
Insulinised animals (0·5 *unit in each case*).							
4	340	0·062	0·79	0·17	72	3·80	1
5	415	"0"	1·63	0·25	204	9·60	2½
6	414	"0"	1·76	0·18	248	6·68	3

It will be seen that the blood-sugars of Nos. 5 and 6 are given as "0." This symbol indicates that the thiosulphate equivalent of the blood-sugar by MacLean's method was less than 0·2 cc. It was frequently noticed in these experiments that a blood-sugar of these vanishing proportions was not necessarily immediately associated with convulsions, and in the above animals there was merely loss of coordination and flaccidity.

In the insulinised animals the liver-glycogen displayed a rise, compared with that of the normal animals, of over 100 % in 1 hour and over 700 % in 3 hours. The muscle-glycogen did not show any significant variations. The values for the liver-fat gave no information at all. The iodine values were 83, 92 and 101 respectively for the controls, and 105, 90 and 118 for the insulinised animals, thus showing no definite change in the degree of saturation of the fatty acids.

Exp. 2. In this experiment the dose of insulin administered was 0·2 unit per animal. Four young rabbits from the same litter, 6 weeks old, were taken. Starvation for 24 hours before experiment.

Controls.

No.	Wt. (g.)	Blood-sugar (g. per 100 cc.)	Glycogen (%) Liver	Glycogen (%) Muscle	Liver-glycogen (mg.)	Remarks
1	565	0·090	0·60	0·06	124	—
2	427	0·110	0·15	0·19	21	—

Insulinised animals (0·2 *unit per animal*).

3	523	0·065	1·40	0·08	272	1 hr. 25 mins. after insulin
4	473	"0"	0·95	0·23	160	2 hrs. after insulin

In this experiment the same effect is seen as far as the liver-glycogen is concerned. The liver-glycogen in No. 1 was unusually high, but in spite of this there could be no doubt as to the very large increase. The results with the muscles were difficult to interpret.

In the next experiment the glycogen deposit at the beginning of convulsions was compared with that in animals after death following convulsions.

Exp. 3. Three young rabbits from the same litter, 6 weeks old. The dose of insulin was 0·5 unit per animal.

No.	Wt. (g.)	Blood-sugar (g. per 100 cc.)	Glycogen (%) Liver	Glycogen (%) Muscle	Liver-glycogen (mg.)	Remarks
1	384	0·11	0·27	0·43	41	Control
2	479	"0"	0·92	0·20	160	Slight convulsion 1¼ hrs. after insulin. Killed 1 hr. 40 mins. after insulin
3	427	"0"	2·03	0·15	292	Violent convulsions 1¾ hrs. after insulin. Died in convulsions 3 hrs. after injection

Again the very large rise in glycogen in the liver is seen, the animal actually dying in convulsions having about seven times as much in its liver as the control. This quantity of glycogen converted into glucose and discharged into the blood-stream would have been ample to avert convulsions and death for a considerable time. Whatever theory be accepted as to the action of insulin, it is quite clear that the glycogen is "locked" in the liver and not even the violent convulsions, with, probably, the stimulation of the adrenals, could in these animals bring about an effective glycogenolysis. The values for muscle-glycogen showed that, at any rate, death was not due to the absence of glycogen, as was suggested by Chaikoff [1925]. There was, however, definitely less glycogen per cent. in the insulinised animals than in the controls. Attention must be drawn to the fact that there was nearly twice as much glycogen in the liver of the animal allowed to die of convulsions as in that of the one killed at the onset of the hypoglycaemic attack. This, we consider, is clear proof that the action of insulin, in so far as it influences the deposition of glycogen, is independent of the convulsions. In fact we formed the impression that the

animal, as far as its glycogenic mechanism is concerned, was divided into two separate systems: one amassing glycogen in the liver and preventing its escape as glucose, and the other depleting such glycogen as was already present in the peripheral tissues. In these young animals the convulsions are never so violent as they are in adult animals and so the fall in muscle-glycogen is not so marked.

In the next experiment it was decided to find how far the very marked effect of insulin on the liver-glycogen could be influenced by the injection of adrenaline. It is well known that adrenaline exerts an antagonistic action to insulin by stimulating glycogenolysis and so preventing hypoglycaemia. The old observation of Gatin-Gruzewska [1906] that adrenaline depletes the glycogen of the body is well known, but a considerable time of action and repeated injections are necessary to produce this effect. Pollak [1909] found that repeated injections of adrenaline into glycogen-free rabbits brought about a deposition of glycogen of the same order as that found in carbohydrate-fed animals.

Drummond and Paton [1904] obtained similar results. Kuriyama [1918] found an increase of glycogen in the livers of fasting rabbits after injections of adrenaline, and Cori [1928] found a marked increase in rats. Muscle-glycogen is found to fall and it has been suggested that adrenaline brings about an increased breakdown of muscle-glycogen to lactic acid, which then can be built up again to glycogen in the liver. In a recent communication on the action of adrenaline in depancreatised dogs Chaikoff and Weber [1928] claim to have shown that it can bring about a marked gluconeogenesis from fat. There is no doubt that adrenaline can produce a very marked hyperglycaemia in animals that have been starved for long periods or otherwise treated until the liver contains only very small amounts of glycogen. If, as seems very probable, these experiments indicate that adrenaline stimulates gluconeogenesis and at the same time glycogenolysis, then its effect on insulin action will depend upon the difference between these two processes.

What we wished to determine was whether any evidence could be obtained that adrenaline influenced the action of insulin directly.

Exp. 4. Four young rabbits from the same litter, 6 weeks old. Starvation 24 hours.

No.	Wt. (g.)	Blood-sugar (g. per 100 cc.)	Glycogen (%)		Liver-glycogen (mg.)	Remarks
			Liver	Muscle		
1	384	0·110	0·27	0·43	41	Control
2	363	0·256	0·38	0·28	49	0·25 mg. adrenaline subcutaneously. Killed 1¾ hrs. later
3	366	"0"	1·56	0·36	190	0·5 unit insulin subcutaneously. Killed at onset of convulsions 1 hr. 35 mins. later
4	364	0·146	1·34	0·20	182	0·25 mg. adrenaline, 0·5 unit insulin at same time. Killed 2 hrs. later

Exp. 5. Two rabbits from the same litter, 6 weeks old. Starved 24 hours.

No.	Wt. (g.)	Blood-sugar (g. per 100 cc.)	Glycogen (%) Liver	Glycogen (%) Muscle	Liver-glycogen (mg.)	Remarks
1	328	0·040	0·34	—	38	Control: blood-sugar unusually low
2	402	0·062	3·86	—	465	2.12 p.m. 0·5 unit 5.38 p.m. 0·5 unit 6.45 p.m. Convulsion 6.55 p.m. Convulsion. 1 mg. adrenaline Killed 2 hrs. later, after complete absence of convulsions for 2 hrs.

It is clear from these experiments that 0·25 mg. adrenaline acting for $1\frac{3}{4}$ hours had no effect on the total liver-glycogen of animal No. 2, experiment 4, although there was hyperglycaemia: but there was a suggestively lower muscle-glycogen. No. 3 showed the usual large increase in liver-glycogen. Animal No. 4 showed that 0·25 mg. adrenaline could not prevent the accumulation of glycogen under the action of insulin, although sufficient sugar was liberated to prevent all hypoglycaemic manifestations. When we consider that, throughout the period of 2 hours after the injections in animal 4, there was a high discharge of sugar from the liver and probably an increased peripheral utilisation, and that in spite of this the accumulation of glycogen was of the same order as in animal 3, it seems not unreasonable to suppose that the adrenaline had contributed to the process of glycogenesis as well as given rise to an increased glycogenolysis.

In experiment 5, 1 mg. of adrenaline, whilst bringing about complete recovery from the insulin convulsions, did not do so by inhibiting the effect of insulin on the liver-glycogen. In fact the increase in liver-glycogen was to about 12 times the control value, in spite of a continuous discharge of sugar for 2 hours.

Up to this point, then, our results indicate that, in the starving young rabbit, insulin brings about a large increase in liver-glycogen and possibly a decrease in muscle-glycogen: further, that the increase in the liver can proceed in spite of the fact that the animal may be approaching death, which could be averted if this glycogen were liberated. It has also been seen that in these young animals death may occur in insulin convulsions from exhaustion, without the glycogen of the muscles having been used up. In addition it has been found that adrenaline in doses sufficient to produce a powerful hyperglycaemia does not necessarily deplete the glycogen in the liver, nor does it inhibit the glycogen increase following the injection of insulin.

Our next series of experiments was carried out with the object of finding what agents could bring about an inhibition of the glycogenesis induced by insulin. From several previous experiments we had satisfied ourselves that anaesthesia with ether or chloroform produces in rabbits a very high and lasting

hyperglycaemia, which involves the whole cardio-vascular system. The question as to whether this effect is due to a stimulus to glycogenolysis or to an inhibition of insulin action presented itself.

It was decided to use ether because of the less severe strain on the vascular system, chloroform being definitely dangerous to such young animals as we were using.

In examining the results obtained it must be understood that in no case in which we have injected insulin into young rabbits has there been any question of the immense rise in liver-glycogen.

Exp. 6. The animals used were 6 weeks old and from the same litter as those in experiment 5. The two rabbits of 5 are included in the following table for comparison. The plan of experiment was to anaesthetise an animal when it developed insulin convulsions and to find the effect of insulin in another animal when it was fully under ether. Muscle-glycogen was not determined.

No.	Wt. (g.)	Blood-sugar (g. per 100 cc.)	Liver-glycogen %	Liver-glycogen mg.	Remarks
1	328	0·040	0·34	38	Control
2	402	0·062	3·86	465	Insulin and adrenaline, *vide* Exp. 5
3	342	"0"	0·69	84	2.13 p.m. 0·5 unit insulin 4.30 p.m. Convulsion Open ether—complete recovery 5.42 p.m. 0·5 unit insulin 6.45 p.m. Convulsion Open ether—complete recovery 7.17 p.m. Convulsion Open ether—complete recovery 7.40 p.m. Killed
4	518	0·104	0·49	84	11.30 a.m. Blood-sugar 0·131 g. per 100 cc. 11.45 a.m. Open ether and kept under 11.46 a.m. 0·5 unit insulin 11.55 a.m. Blood-sugar 0·122 g. per 100 cc. 12.45 p.m. Blood-sugar 0·117 g. per 100 cc. 1.30 p.m. 0·5 unit insulin 1.35 p.m. Blood-sugar 0·077 g. per 100 cc. 3.15 p.m. Killed after 3½ hrs. anaesthesia

The first point which emerges from this experiment is that the insulin convulsions were completely stopped by the ether anaesthesia. In the case of animal 4 the onset of convulsions was entirely prevented. With animal 3 the anaesthesia was light, so that convulsions occurred when it wore off somewhat: but in the case of 4 the continuous and deep anaesthesia almost entirely inhibited the action of the insulin, thus, in 3½ hours after the two injections of 0·5 unit each, the blood-sugar had only fallen from 0·131 to 0·104 g. per 100 cc. Further, in neither case did there occur the very large rise in liver-glycogen which occurs invariably after insulin alone. There was, it is true, over twice as much glycogen in the livers of 3 and 4 as in the control, but this is a very small effect compared to that, for example, in No. 2.

We conceive that in animal 3 the glycogen content of the liver was rising and would have continued to do so had the animal been allowed to go on living

for as long as it could under the circumstances. The action of the insulin would come in as the effect of the ether wore off.

In animal 4, on the other hand, the continuous anaesthesia tends to promote a continuous flow of sugar from the liver whilst the insulin ineffectually antagonises this by exerting a "locking" action on the liver. This effect is different from that of adrenaline, for, as we have seen in experiments 4 and 5, an injection of this substance sufficient to prevent convulsions produces hyperglycaemia and does not prevent the insulin from promoting a large deposition of glycogen in the liver. It seems to us that the effect of the anaesthetic is due to a toxic action on the liver cells, making it difficult for them to elaborate glycogen from exogenous or endogenous carbohydrate.

In animal 3 there must have been at 4.30 p.m. a large increase in liver-glycogen over the value at the outset, so that the anaesthesia must have liberated it again, this happening each time the convulsed rabbit was given ether. In animal 4 there did not occur any considerable hyper- or hypo-glycaemia throughout the $3\frac{1}{2}$ hours of the experiment, hence the inability to lay down any largely increased amount of glycogen must have been due to some effect on the liver itself.

The very powerful liberation of sugar which takes place in rabbits under ether is shown in the following experiment on an adult animal.

Time after ether anaesthesia (mins.)	Blood-sugar, g. per 100 cc.				
	0	10	40	48	58
Peripheral vein	0·129	0·193	0·327	0·383	0·383
Inferior Vena Cava	—	—	0·330	0·340	0·378
Portal vein	—	—	0·293	0·327	0·362

The fact that this effect was absent in animal 4, experiment 6, shows that glycogenolysis was at its normal level and hence the failure to lay down any greatly increased quantity of glycogen localises the inhibition in the liver itself.

The failure of insulin to produce its typical effects in certain cases of very severe diabetes may be analogous to this effect of ether. The intense acidosis and poisoning with the toxic products of abnormal metabolism may produce this antagonism to a successful action of insulin by rendering the cell incapable of forming glycogen. Thus Sevringhaus [1926] found that injections of sodium acetoacetate into fasting rabbits retarded the blood-sugar-lowering action of insulin. Koehler [1926] found that in diabetics with well-marked acidosis (blood-p_H 6·8 to 7·2) doses of 100 to 300 units in 24 hours had no marked effect. The partial loss of tolerance to carbohydrate, induced by the ingestion of NH_4Cl, $CaCl_2$ or $NaHCO_3$ in big doses, is probably due to a similar toxic condition of the hepatic cell. The same is probably true of the intolerance to carbohydrate arising in starvation.

If this view of the action of ether is correct, its effect in preventing insulin convulsions and the accumulation of glycogen in the liver becomes understandable.

Adrenaline acts by liberating hepatic carbohydrate, at the same time probably increasing gluconeogenesis, and so does not, within the period of our experiments, interfere with the action of insulin in promoting an accumulation of liver-glycogen, although the blood-sugar may be maintained at high levels. Ether, on the other hand, prevents insulin from exerting its typical effect, by making it difficult or impossible for the liver cells to transform sugar to glycogen.

The next matter which was examined was the question as to how far the action of insulin could be influenced by convulsions. There seems to be no doubt that convulsions in a muscle deplete its glycogen, although there is some evidence that a muscle can respond to induction shocks even when its glycogen content has been reduced to vanishing proportions by injection of convulsants [Olmsted and Harvey, 1927]. Now in our young animals the convulsions induced by insulin are not powerful enough to deplete either muscle- or liver-glycogen. What we wished to determine was whether convulsions produced by, say, strychnine sulphate could in the presence of insulin prevent the accumulation of glycogen in the liver. Two types of experiment were possible: (1) to induce strychnine convulsions during the action of insulin, and (2) to inject insulin during and between strychnine convulsions.

Exp. 7. Seven young rabbits from the same litter, 6 weeks old. Starvation 24 hours before experiment in each case.

No.	Wt. (g.)	Blood-sugar (g. per 100 cc.)	Glycogen (%) Liver	Glycogen (%) Muscle	Liver-glycogen (mg.)	Remarks
1	451	0·113	0·59	0·30	78	Control
2	448	0·090	0·32	0·14	56	2.48 p.m. 0·2 mg. strychnine 2.52 p.m. Convulsion—typical 3.20 p.m. Died after 28 mins. of convulsions
3	457	0·109	0·26	0·19	50	1.35 p.m. 0·05 mg. strychnine 2.06 p.m. 0·10 mg. strychnine 2.36 p.m. 0·10 mg. strychnine 2.54 p.m. 0·20 mg. strychnine 2.57 p.m. Convulsion 3.34 p.m. Died after 37 mins. of convulsions
4	461	0·058	1·37	0·16	230	2.50 p.m. 0·5 unit insulin 5.02 p.m. 0·2 mg. strychnine 5.12 p.m. Strychnine convulsions 5.13 p.m. Died—11 mins. after injection of strychnine
5	442	0·113	1·90	0·15	374	2.00 p.m. 0·5 unit insulin 3.05 p.m. Hypoglycaemic reaction 3.21 p.m. Insulin convulsion with recovery 3.22 p.m. 0·2 mg. strychnine 3.27 p.m. Insulin convulsion with recovery 3.34 p.m. Strychnine convulsions—very violent 3.39 p.m. Died—17 mins. after injection of strychnine

No.	Wt. (g.)	Blood-sugar (g. per 100 cc.)	Glycogen (%) Liver	Glycogen (%) Muscle	Liver-glycogen (mg.)	Remarks
6	454	0·110	0·46	0·15	79	5·28 p.m. 0·2 mg. strychnine 5.50 p.m. Violent convulsions 5.51 p.m. 0·5 unit insulin 6.14 p.m. 0·2 mg. strychnine 6.30 p.m. Died—62 mins. after first dose of strychnine and 39 mins. after injection of insulin
7	453	0·056	0·26	0·21	46	6.59 p.m. 0·2 mg. strychnine 7.32 p.m. Convulsion with recovery 7.53 p.m. 0·15 mg. strychnine 8.04 p.m. Strychnine convulsion. 0·5 unit insulin 8.40 p.m. Strychnine convulsion ceased 8.56 p.m. 0·2 mg. strychnine 9.06 p.m. Intermittent convulsion 9.21 p.m. Killed—after 3 hrs. 22 mins. strychnine action and 1 hr. 17 mins. insulin action

Several facts emerge from this experiment. First, it is clear that these young animals can die after convulsions lasting over 30 minutes without losing all the glycogen from the liver or muscles, or, indeed, depleting it to any considerable extent. This is probably due to the fact, already mentioned, that the convulsions are not as violent as in an adult animal and that the young animals die of exhaustion. Nos. 2 and 3 illustrate this point. It is also to be observed that the period of convulsion was in both animals about half an hour.

In the cases of Nos. 4 and 5 the strychnine was injected when they were in a definitely hypoglycaemic condition and it was found that both animals died extremely rapidly. The effect was very striking, in No. 4 death followed in 1 minute after the onset of convulsions and in No. 5 in 5 minutes. In Nos. 4 and 5 the hypoglycaemia had been remedied by the strychnine convulsions, the values being 0·058 % and 0·113 % respectively. Increases were observed, moreover, in the glycogen in the liver of the same order as that when insulin acts alone, and the muscles contained amounts not significantly different from those of other young animals. Our attitude to the muscle figures is governed by the principle that we are in search of big effects and are loath to base hypotheses on small absolute changes. One thing, however, is clear, namely, that these animals did not die from hypoglycaemia or from depletion of muscle- and liver-glycogen. Of course in both cases the strychnine convulsions only lasted from 1 to 5 minutes, so that one could hardly expect much effect, but the fact that death followed so quickly seems to point to some hypersensitivity to the alkaloid in the insulinised animal.

When, however, we examine the results obtained from animals 6 and 7 the picture is quite different in several respects. In animal 6, 0·2 mg. strychnine brought about violent convulsions in 22 minutes. 0·5 unit insulin was at once injected and the animal had no further convulsion during the succeeding 24 minutes. An additional 0·2 mg. strychnine was then injected and the

animal died in 16 minutes, that is, 62 minutes after the injection of the first dose of alkaloid and after the insulin had been acting for 40 minutes.

In animal 7, 0·2 mg. strychnine induced convulsions in 33 minutes, but recovery soon occurred. Another dose of 0·15 mg. induced violent convulsions in 11 minutes, when 0·5 unit insulin was injected. Convulsions of the strychnine type continued for 36 minutes and then ceased. After 16 minutes without any convulsion a further 0·2 mg. strychnine was injected and in 10 minutes violent intermittent convulsions set in. The animal was killed 15 minutes later, that is, 3 hours, 22 minutes after the first injection of strychnine and 1 hour, 17 minutes after the injection of insulin. In neither of these animals did a decided hypoglycaemia occur, the heart blood-sugars being 0·110 % and 0·056 %. As seen from animals 2 and 3, strychnine does not bring about a hyperglycaemia, so there is no analogy to the actions of adrenaline and ether, although the convulsions may conceivably stimulate the adrenals to some extent. Examining the results further, it is readily seen that the glycogen contents of both liver and muscle showed no significant departure from those of untreated animals, thus contrasting strikingly with the results in the cases of animals 4 and 5.

It appears then that during the violent convulsions following strychnine, insulin does not bring about its typical effect. Death, when it occurs in these conditions, does not result from the action of insulin but from that of the alkaloid.

It is tempting to imagine some interference arising from the action of the strychnine on the spinal cord, but much further evidence is necessary. There is, indeed, some evidence that insulin exerts its effect *via* the cord, but it is still only in the presumptive stage.

Discussion.

We take the provisional view that the action of insulin is to prevent the discharge of glycogen from the liver. We conceive that the process of absorption into the liver of glycogen-producing substances goes on from the gut and the blood as freely in the starving insulinised animal as in the control, but for some reason yet to be discovered the process of glycogenolysis is prevented. Peripheral utilisation continues and the blood-sugar reaches levels at which symptoms appear. This "locking" effect on liver-glycogen can be readily and strikingly demonstrated in young fed rabbits as well as starving animals.

Exp. 8. Two young rabbits from the same litter, 6 weeks old. Taken off food during the period of experiment only.

No.	Wt. (g.)	Blood-sugar (g. per 100 cc.)	Glycogen (%)		Liver-glycogen (g.)	Remarks
			Liver	Muscle		
1	297	0·156	9·94	0·66	1·39	Control
2	373	"0"	9·26	0·31	2·04	1.05 p.m. 0·5 unit insulin 3.00 p.m. 0·5 unit insulin 5.25 p.m. 0·5 unit insulin 7.05 p.m. Convulsion with recovery 7.15 p.m. Killed

Exp. 9. Two young rabbits from the same litter, 6 weeks old. Taken off food during the period of experiment only.

No.	Wt. (g.)	Blood-sugar (g. per 100 cc.)	Liver-glycogen (g.)	Remarks
1	542	0·143	1·56	Control
2	517	"0"	1·03	1.10 p.m. 0·5 unit insulin 4.30 p.m. 0·5 unit insulin 5.15 p.m. Convulsion 5.30 p.m. Convulsion 5.40 p.m. Convulsion 5.43 p.m. Killed

Here we have animals, No. 2 in each experiment, which are *in extremis* for the want of blood-sugar and which possess between 1 and 2 g. of glycogen in the liver. Experiment 8 shows also the very much lower muscle-glycogen of the insulin-treated animal. The blood contained practically no copper-reducing substance and the liver was incapable of yielding any to it.

That in the fed animal it takes much longer and much larger doses of insulin to produce hypoglycaemia than in the starved animal we conceive as due to the difficulty of increasing the glycogen in a liver already highly charged with it. Thus the removal of blood-sugar would depend largely on peripheral utilisation. In the last two experiments there did not occur any significant change in the liver-glycogen, the sum of the two controls being 2·95 g. and in the insulinised animals 3·07 g.; so that if we accept the idea that insulin "blocks" the release of glycogen we must also be prepared to consider, in the fed animal, the probability of a factor relating to the regulation of absorption, because the gut in the fed animals was full of carbohydrate and other food in process of digestion. It seems reasonable to suppose that when the liver-glycogen reaches a certain level it is impossible to raise it further, so that the glycogenic function would become a self-limiting process. Insulin acting in these circumstances would block the outward delivery of sugar and the liver itself would inhibit any further uptake of carbohydrate from other sources. The probable existence of a limiting glycogen content in the liver, characteristic for each animal, can be demonstrated by an experiment using rabbits fed on a diet containing an excess of carbohydrate for varying periods. The following was such an experiment.

Exp. 10. Seven adult rabbits were used. Two were kept as controls on their usual diet of bran, oats and green vegetables. The remaining five were given carrots and greens for the periods indicated. They were then killed and the livers and muscles analysed for glycogen and fat. The fat was estimated to see if the excessive carbohydrate was perhaps stored as fat.

	Glycogen (%)		Fatty acid (%)		
No.	Liver	Muscle	Liver	Muscle	No. of days on carrots
1	2·60	0·40	—	1·65	Control
2	0·40	0·26	4·03	2·02	"
3	6·30	0·41	2·75	1·49	2
4	11·29	0·05	2·07	1·29	7
5	7·54	0·21	2·74	1·31	12
6	4·90	0·11	2·62	1·68	15
7	8·60	0·06	2·68	1·20	31

We take these figures to show that on such a diet the liver-glycogen cannot be made to exceed about 11 %, and although we have not yet tried this in adult rabbits we think that the injection of insulin would not bring about any higher level than this. The view, then, put forward is that the absorption of sugar from the gut or the formation of sugar by gluconeogenesis is governed by the amount of glycogen already present in the liver. Insulin, by "locking" the liver-glycogen, tends to prevent both the processes of absorption and of gluconeogenesis. It is not, we suggest, the insulin as such which does this, but it is the result of the accumulation of glycogen. We conceive that it is the actual presence of the colloid glycogen which imposes a self-limiting action on the liver.

On this view the action of insulin might be explained as due to two factors: (*a*) the blockage of carbohydrate in the liver, and (*b*) the self-limiting mechanism of the liver.

Let us consider briefly the application of this view to the cases of (1) the starving animal, (2) the fed animal, and (3) the diabetic.

(1) The starving animal, with its small amount of glycogen in the liver, keeps up its blood-sugar by absorption from the gut and later by gluconeogenesis from protein and fat (the evidence for the latter source of carbohydrate is doubtful and will be the subject of a further communication). The endogenous internal secretions of the pancreas, thyroid and adrenal control this process so that an optimum balance is struck, conserving the body tissues as far as possible. Insulin given to such an animal blocks the release of carbohydrate from the liver, but offers no obstruction to absorption and gluconeogenesis, because the liver-glycogen is still far from the limiting value. The result is a large rise in liver-glycogen. And, as we have seen, this occurs even when adrenaline is acting strongly. The necessary and sufficient condition for this action of insulin is a healthy liver.

(2) In the animal which already possesses a high liver-glycogen the discharge of sugar into the blood is as steady as and not at a much higher level than in the starving animal. This discharge is obviously not accompanied by an equal replenishment, otherwise the liver-glycogen would not fall as we know it does. This, in our view, is due to a limitation of absorption and stoppage of gluconeogenesis. In such an animal insulin, although blocking the discharge from the liver, does not secondarily bring about much change in the liver-glycogen, because the high glycogen content has imposed the self-limiting process on the liver. Thus the animal goes into hypoglycaemia much more slowly, since the disappearance of peripheral sugar will depend mostly on peripheral utilisation. This continues until the animal may die in convulsions without liberation of sugar from the liver.

(3) In the diabetic subject absorption of carbohydrate goes on rapidly from the gut because of the low glycogen values in the liver and the loss of blockage power, due to the deficiency of insulin. Gluconeogenesis proceeds at a rate which is much greater than in the starving animal because of the loss

in conserving power. The R.Q. is low because of the high degree of gluconeogenesis counterbalancing the high R.Q. of carbohydrate utilisation. The height of the R.Q. under any circumstances must be the algebraic sum of these processes. Insulin, provided the liver cells are not damaged and are capable of elaborating glycogen, blocks the discharge of carbohydrate from the liver, the liver-glycogen increases until as a consequence excessive absorption is stopped and gluconeogenesis is inhibited. The R.Q. now rises because the latter process is controlled. The condition for effective action is again the condition of the liver cells.

The whole problem, therefore, resolves itself into how insulin "blocks" the liver-glycogen.

There does not appear to be any evidence that insulin increases metabolic rate more than can be brought about by normal effective metabolism. It has always seemed anomalous that the diabetic should have a more or less normal metabolic rate, and that even when his metabolic processes are controlled by means of insulin the total metabolism shows no significant increase. This, it seems to us, can only be explained by a readjustment of the partition of energy among fat, protein and carbohydrate, which comes about by prevention of uncontrolled release of carbohydrate from the liver and the consequent depression of gluconeogenesis.

SUMMARY.

1. Young rabbits, 6 to 10 weeks old, of the same litter, are well adapted to comparative experiments with insulin. When starved for 24 hours they show a very satisfactory uniformity in their liver-glycogen. The average of twelve such animals from various litters was 50 mg. glycogen per liver.

2. Comparing the glycogen content of such rabbits, starved for 24 hours and treated with insulin, with that of controls from the same litter, there invariably occurred a very large rise in liver-glycogen. The average for twelve such animals was 237 mg.

3. Young rabbits treated with insulin may die in hypoglycaemic convulsions and still possess a very much increased liver-glycogen and a very considerable muscle-glycogen.

4. Adrenaline, whilst relieving hypoglycaemia, does not interfere with the essential action of insulin in increasing liver-glycogen.

5. Light ether anaesthesia relieves the convulsions due to hypoglycaemia. During continuous deep anaesthesia insulin cannot bring about the usual very large increase in liver-glycogen. This is considered to be due to a toxic action on the liver cell.

6. The hypoglycaemic animal is very susceptible to strychnine and dies in a very much shorter time than the normal animal. Continuous strychnine convulsions can prevent the typical action of insulin in promoting an increased glycogen content of the liver.

7. In the fed animal insulin does not bring about an increase in liver-glycogen and the animal goes into hypoglycaemic convulsions with large quantities of glycogen in the liver. Muscle-glycogen is definitely lowered.

8. These results are discussed and a theory of insulin action is suggested, *viz.* that the immediate effect of insulin is to inhibit the release of liver-glycogen. All other effects are regarded as secondary to this. The application of this view to certain cases is discussed.

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XIV. AN APPLICATION OF THE METHOD OF HAGEDORN AND JENSEN TO THE DETERMINATION OF LARGER QUANTITIES OF REDUCING SUGARS.

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The method of Hagedorn and Jensen [1923] has become widely used for the estimation of reducing sugar in blood. The method depends upon the fact that potassium ferricyanide is reduced to ferrocyanide when heated in alkaline solution with certain reducing substances. In the Hagedorn and Jensen procedure the ferrocyanide formed in this way is precipitated as the double potassium zinc salt, and the residual ferricyanide is estimated by adding an excess of potassium iodide and acidifying. The ferricyanide is reduced quantitatively by the iodide, and equivalent iodine is liberated according to the equation:

$$2H_3Fe(CN)_6 + 2HI = 2H_4Fe(CN)_6 + I_2.$$

The iodine is titrated with thiosulphate.

This method based on ferricyanide as the substance reduced has an inherent advantage over copper methods which will be referred to later. In the original form it was devised for estimating the reducing sugar in the filtrates from 0·1 cc. samples of blood. The capacity is accordingly very small, the upper limit being 0·385 mg. of glucose. Small volumes of solutions are used, and the essential reactants are present in low concentration so that the introduction of impurities may cause relatively large errors. For the purposes of my own work the range of the original method was inconveniently narrow, and the present modification makes possible the estimation of about ten times as much sugar. The new procedure has been standardised for maltose as well as for glucose. No essential change from the original method has been made, except as regards the concentrations and volumes of the various reagents employed.

The following solutions are used in the modified method. The chemicals used were of the "Analytical Reagent" quality, as supplied by the British Drug Houses, Ltd., unless otherwise stated.

Solution A.

Potassium ferricyanide	...	...	8·25 g.
Anhydrous sodium carbonate		...	10·6 g.

Make up to 1 litre with distilled water. This solution should be kept for 2 or 3 days before use and it must be stored in a bottle provided with an opaque jacket. In making the solutions for the standardisation data the potassium ferricyanide was twice recrystallised but as no significant difference is found when the untreated "A.R." product is used, this is not considered necessary.

Solution B.

Potassium iodide	...	...	12·5 g.
Zinc sulphate	...	...	25·0 g.
Sodium chloride	...	...	125·0 g.

Make up to 500 cc. with distilled water. Traces of iodine appear in this solution on storing. This iodine should be removed before using by filtering through two thicknesses of filter paper.

Solution C.

5 cc. glacial acetic acid, diluted to 100 cc. with distilled water.

Solution D.

Soluble starch solution, saturated with sodium chloride. 1 g. Merck's soluble starch in about 20 cc. cold water is washed into 60 cc. of boiling water. This is boiled for 2 minutes, 20 g. sodium chloride are added and the solution is allowed to cool. It is then made up to 100 cc. This solution keeps for several months.

Solution E.

An approximately *N*/75 sodium thiosulphate solution which is used in a 10 cc. burette, graduated into 0·02 cc. divisions. In practice it is found convenient to make up about 10 litres of this solution (containing 3·33 g. sodium thiosulphate per litre) using boiled-out water. It is then stored in a bottle, protected from atmospheric carbon dioxide by a soda-lime tube, and the solution is run through a siphon to the burette. When stored in this way the solution keeps well. At first it should be standardised each day but after the first week it need only be standardised at intervals of 3 or 4 days.

The thiosulphate solution is most conveniently standardised against a potassium iodate solution. This solution is made up by weighing out carefully a quantity of about 0·80 g. of desiccated potassium iodate and making it up to a volume of 1 litre in a volumetric flask standardised at the temperature obtaining. A 5 cc. pipette of slow delivery is standardised by weighing four or five deliveries of water at a known temperature. (The tip should, of course, be drained against the side of the collecting vessel for a standard time.) With this pipette 5 cc. of the standard iodate solution are run into a boiling-tube. To this are added 5 cc. of a 2 % solution of potassium iodide and then it is acidified by the addition of 3 cc. of 5 % acetic acid (solution C). The iodine liberated is titrated with the thiosulphate solution, adding a drop of the

starch solution D when the colour becomes a pale yellow, and titrating to the disappearance of the blue colour. The volume of thiosulphate required when the normality is 0·01333 ($N/75$) is about 9 cc. The value for the normality is found from the expression

$$\frac{\text{g. K iodate per litre} \times \text{vol. of pipette (cc.)}}{35{\cdot}67 \times \text{vol. of thiosulphate required (cc.)}}$$

The results from this method were found to give exact agreement with the method described by Cole [1928] in which the thiosulphate is standardised against a standard acid.

Procedure for determination of sugar.

The estimations are carried out in boiling-tubes, 1 × 7 in. Glass bulbs with about an inch of tubing left attached are used as covers for the boiling-tubes.

5 cc. of solution A (alkaline ferricyanide) are pipetted into a boiling-tube, allowing the pipette to drain a standard time. To this are added 5 cc. of the solution whose reducing power is to be determined. (Note: if less than 5 cc. is added, sufficient water is added to make up the difference in volume.) A water blank is now made up by pipetting 5 cc. of solution A into a second tube and adding 5 cc. of distilled water. It is important that any drops of liquid adhering to the walls of the tubes should be mixed in, and this is best done by inclining the tube and slowly rotating it.

The tubes are now covered with glass bulbs and placed for 15 minutes in a boiling water-bath 2 or 3 in. deep. After this they are cooled for 3 minutes in cold running water.

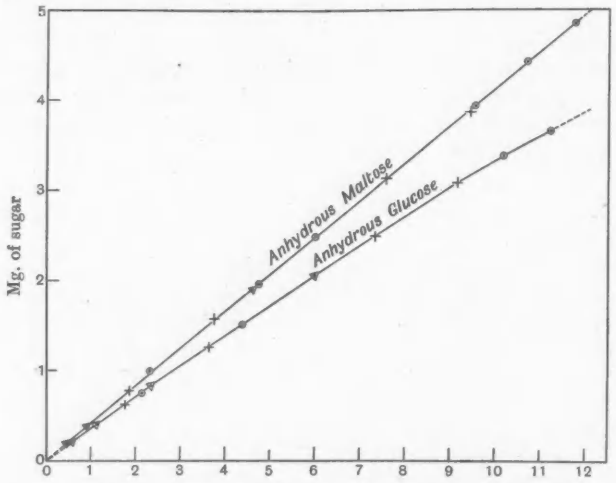
The remaining operations are concerned with the estimation of the residual ferricyanide. 5 cc. of solution B are added. This is conveniently run in from a rapid pipette as the volume need not be precise. A white flocculent precipitate appears and at the same time iodine is set free. 3 cc. of solution C are now added (also from a rapid pipette). The reduction of the ferricyanide is now completed and the iodine liberated is titrated against the standardised thiosulphate.

The titration is carried out in the same boiling-tube. Mixing during the titration is effected by shaking the tube with a circular motion. The titration should be carried out in a regular manner. Thiosulphate is run in slowly until the liquid has a pale yellow colour; 3 drops of the starch indicator are then added and thiosulphate is added drop by drop until the sudden disappearance of the blue colour[1]. The end-point is very sharp and it is advantageous to fit the burette with a fine tip which has been coated with paraffin wax as this makes the drops smaller and prevents the thiosulphate from wetting the tip.

[1] An "overshot" end-point may be regained by adding counted drops of iodine (about $N/75$ in KI) from a fine dropping pipette until the blue colour returns, titrating to the end-point with thiosulphate, and deducting from the reading the thiosulphate equivalent of the added iodine which is found by repeating the operation.

The difference between the water blank value (WB) and the reading obtained with the experimental solution (R) gives the thiosulphate equivalent of the ferricyanide reduced by the experimental solution. These reduction values have been determined for different amounts of added reducing sugars, maltose and glucose, and the results are shown in the standardisation graph, Fig. 1. For convenience in calculation the results are given in terms of $N/100$ thiosulphate, although approximately $N/75$ is used in the titration. Thus if the normality of the thiosulphate used for a particular estimation is 0·01351, then the $N/100$ thiosulphate equivalent of the ferricyanide reduced is (WB − R) × 0·01351 cc. The amount of sugar corresponding to this reduction is either read from the graph, or in the case of maltose it may be calculated from a factor.

In practice the water blank value need be determined only once or twice a day. Estimations can be done conveniently in batches of five or six and in this way it is possible to make 18 estimations an hour when facility in the technique has been acquired.



cc. $N/100$ thiosulphate equivalent to ferricyanide reduced (WB – R)

Fig. 1.

The heating period for reduction.

In order to follow the reduction after different periods of heating, five boiling-tubes were set up each containing 5 cc. of solution A and 5 cc. of a maltose solution containing 2·5 mg. These were placed in the boiling water-bath and allowed to remain for 5, 10, 15, 20, and 30 minutes respectively. On removal each tube was cooled for 3 minutes and the residual ferricyanide was estimated in the usual way. The results are given in Fig. 2 in which the amounts of ferricyanide reduced (WB − R) are plotted against time of heating.

It is evident that during the first 15 minutes most of the reduction has taken place. Actually the ferricyanide reduced at 20 minutes is only 1·5 % more than at 15 minutes (with this amount of sugar present). The 15-minute period was adopted as the standard heating time.

The standardisation graphs.

Maltose.

The maltose used was a sample of Kahlbaum's pure maltose which had been recrystallised three times from 60 % alcohol and then dried in warm air. For this specimen I am indebted to Mr S. W. Cole of the Biochemical Institute, Cambridge. When dried *in vacuo* over concentrated sulphuric acid for 2 hours the sugar lost 0·1 % in weight. The optical activity was $[\alpha]_D^{20^\circ} + 130{\cdot}7^\circ$ using a solution of about 9 %. This falls within the range of published values for maltose monohydrate, although it is somewhat greater than the average given by Tollens [1914] as + 129°–130°.

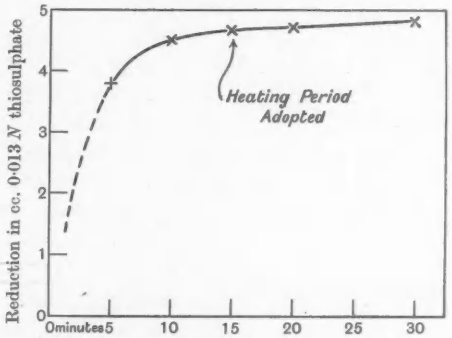


Fig. 2.

Owing to the difficulty of preparing anhydrous maltose, this was not attempted, but the data have been obtained from the hydrate and calculated in terms of the anhydrous sugar by multiplying weights of the hydrate by the factor 342/360.

Maltose solutions of known strength were prepared by weighing out quantities of the sugar and making up to a known volume in a standardised volumetric flask. A set of standardised Ostwald pipettes, from 0·5 to 5·0 cc. capacity was used to measure out portions of these solutions into boiling-tubes containing the standard 5 cc. of solution A, and the amount of ferricyanide reduced after 15 minutes' heating was determined in the way described. These data have been used to construct the standardisation graphs (Fig. 1). The reliability of the procedure may be seen from the fact that the points which were obtained from three different maltose solutions all lie very nearly on the same line. The experimental data on which these points are based are given in an appendix.

In the case of maltose the relationship between ferricyanide reduced and the amount of sugar is a linear one. 1 cc. of $N/100$ thiosulphate is equivalent to 0·414 mg. anhydrous maltose over the entire range and it is therefore convenient to employ this factor for the conversion of reduction values (WB − R) into maltose.

Glucose.

The glucose used was a sample of "Dextrose A.R." (British Drug Houses). It was desiccated *in vacuo* over concentrated sulphuric acid for 48 hours by which time it was found to have attained a constant weight. The specific rotation of this desiccated sugar was $[\alpha]_D^{20^\circ} + 52{\cdot}2^\circ$ in a 10 % solution. Theoretical value for anhydrous glucose + 52·5°.

The glucose standardisation data were obtained as for maltose. The points from three solutions lie very nearly in the same line, but the relationship is not linear here as it is for maltose. This difference between the two sugars was investigated. The reduction progress curves for small and large amounts of glucose or maltose have different forms. When plotted as percentages of the reduction after 30 minutes' heating against time of heating, the curves intersect: those for maltose at 15 minutes, for glucose at 10. Consequently after the standard heating (15 minutes) the reduction by maltose is proportional to the maltose added, but this is not so for glucose since at 15 minutes the percentage progress curve for a small amount of sugar lies below the curve for a large amount. For glucose then, reduction values are most conveniently converted into sugar values by reading from a graph. Table I gives figures for constructing this.

Table I. *The glucose standardisation graph.*

Mg. glucose	cc. $N/100$ thiosulphate	Mg. glucose	cc. $N/100$ thiosulphate
0·20	0·58	2·00	3·40
0·50	1·45	2·50	7·38
0·80	2·32	3·00	8·93
1·00	2·91	3·50	10·55
1·50	4·40	3·80	11·53

Note. These figures have been read at convenient intervals from a large scale graph of the experimental data (Fig. 1).

THE STABILITY OF THE ALKALINE FERRICYANIDE (SOLUTION A).

It was thought important to investigate the keeping qualities of solution A in view of the fact that certain authors have suggested that the alkaline ferricyanide solution of the original Hagedorn and Jensen method should not be kept long. One modification of the original method has been proposed in which the ferricyanide and the alkali are made up separately, to be mixed immediately before the estimation (Pattersen, quoted by Cole [1928]). Accordingly lots of solution A were made up at different times and stored in black-jacketed bottles at room temperature, to be compared at a later date. The results are given in Table II for the reducing values found for 5 cc. of a maltose

solution containing 3·86 mg. maltose, using these solutions. The change after the second day is very slight and it must be concluded that storing the solution for 3 months causes practically no deterioration. During the first 2 days of storage, however, there seems to be an appreciable change in the reduction, solution A should therefore be prepared 2 or 3 days before using. The solution A used in the standardisation estimations had been made up 6 to 10 days.

Table II.

Time of storing solution A	Values in cc. 0·01290 *N* thiosulphate		
	(*a*) Water blank	(*b*) With 3·86 mg. maltose	(*c*) Thiosulphate equivalent of reduced ferricyanide
10 min.	9·59	2·39	7·20
2 hrs	9·61	2·40	7·21
2 days	9·61	2·32	7·29
8 ,,	9·64	2·34	7·30
28 ,,	9·60	2·35	7·25
34 ,,	9·54	2·21	7·33
90 ,,	9·63	2·30	7·33

THE EFFECT OF DISSOLVED OXYGEN.

I have found previously[1] that when copper methods are used a considerable error may be involved in estimating the reducing power of sugar solutions with different amounts of dissolved oxygen. Thus, with the Shaffer and Hartman micro-method, two samples of the same sugar solution, one free from oxygen and the other saturated with oxygen, give reducing values differing by as much as 25 %. The lower the concentration of dissolved oxygen, the higher becomes the reducing value obtained. This was shown to be due to the re-oxidation of cuprous to cupric copper by dissolved oxygen in the early stages of the reduction heating.

The Hagedorn and Jensen method (and the present modification) are found to be entirely free from this defect, as is shown in the following data, which were obtained by taking two portions of a solution of maltose, saturating one with oxygen by bubbling the gas through it and washing the other with a stream of pure nitrogen. The reducing values were then determined.

Mg. maltose found in 5 cc. sample.

	(*a*) Free from oxygen	(*b*) Saturated with oxygen
Solution 1	4·05	4·02
,, 2	1·54	1·54

Since the values found are very nearly the same it is evident that there is no appreciable re-oxidation of the ferrocyanide by dissolved oxygen under the conditions of the procedure. It is much safer, therefore, to use a ferricyanide method when dealing with solutions whose oxygen equilibrium may have been disturbed by any previous treatment such as boiling, filtering under reduced pressure, or washing with a gas stream other than air.

[1] Hitherto unpublished.

The effect of the presence of starch.

The present method was worked out primarily for the purpose of following the progress of sugar production during the hydrolysis of starch; it was necessary therefore to find out whether the presence of an excess of starch interfered with the final iodine titration. It is found that although the titration is slightly more difficult in that there is a tendency to overshoot the end-point, a little practice overcomes this difficulty. The stirring must be more vigorous and towards the end of the titration the thiosulphate must be added more slowly. Experiments were done in which known amounts of maltose were added to solutions of soluble starch and amylose of various concentrations. The latter were undialysed and had small reducing powers themselves. Values found for added maltose, subtracting the reduction due to the starch or amylose, were in all cases in close agreement with the amounts added.

Summary.

The range of the Hagedorn and Jensen sugar method has been extended to enable the estimation of about ten times the amount of reducing sugar. The solutions and procedure for the modified method are described and standardisation data are given for glucose and maltose. An important advantage over copper methods is pointed out in the fact that variations in the amounts of dissolved oxygen in sugar solutions do not affect the reducing values arrived at by the ferricyanide method.

In conclusion I wish to express my thanks to Mr S. W. Cole of the Biochemical Institute, Cambridge, for his very generous help and criticism.

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APPENDIX.

The standardisation data.

Water blank values: 9·62, 9·63, 9·625, 9·605, 9·615, 9·61, 9·62, 9·61, 9·62 cc.
Thiosulphate used: 0·01290 *N*.

Mg. glucose	3·71	3·39	3·08	2·49	2·05	1·50	1·25	0·829	0·749	0·622	0·414	0·213
Ferricyanide reduced*	9·72	7·90	7·11	5·69	4·64	3·42	2·83	1·83	1·69	1·39	0·88	0·46
(WB – R)	9·68	7·92	7·12	5·70	4·64	3·40	2·83	1·84	1·69	1·37	0·89	0·48
Mg. maltose	4·86	4·44	3·93	3·86	3·13	2·47	1·96	1·89	1·56	0·980	0·780	0·382	0·197
Ferricyanide reduced*	9·12	8·29	7·40	7·32	5·86	4·65	3·69	3·59	2·92 2·94	1·81	1·46	0·70	0·39
(WB – R)	9·09	8·28	7·39	7·31	5·92	4·66	3·69	3·57	2·92	1·82	1·47	0·71	0·37

* Expressed in terms of cc. of 0·01290 *N* thiosulphate.

XV. THE NATURE OF THE UNSAPONIFIABLE FRACTION OF THE LIPOID MATTER EXTRACTED FROM GREEN LEAVES.

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(*Received January 1st, 1929.*)

In an investigation now being carried out by E. M. Hume at the Lister Institute, the unsaponifiable matter of the lipoid substances extracted from spinach leaves was being prepared by her to supply the vitamin A in a certain diet. During the course of this preparation we noticed that a white crystalline substance readily separated when this unsaponifiable matter was being taken up with hot alcohol, and we therefore decided to investigate its nature.

Method of preparation. The spinach leaves were dipped for a few seconds into boiling water before drying at 37° under a fan. The preliminary dipping expels air from the tissue and leaves so treated are crisp and green when dry. They were then powdered and extracted with light petroleum (B.P. 40–60°) at the laboratory temperature, being shaken for part of the time of each extraction. The petroleum was removed by evaporation and the residue dried to constant weight, dissolved in ether and a 5 % alcoholic solution of sodium ethoxide added. After standing overnight part of the alcohol was removed by evaporation, water added and the aqueous solution extracted with ether; an orange brown substance was obtained from this ethereal extract. Working in this manner, 2 kg. of spinach gave 230 g. of dried leaf tissue and, after this had been eleven times extracted, each time with 2 litres of light petroleum, 18·4 g. of lipoid substance were separated containing 4·6 g. of unsaponifiable matter. The latter is an orange brown solid giving with concentrated sulphuric acid the blue colour characteristic of carotene. The sterol content was determined by precipitation with digitonin, and was found to be less than 5 %. The sterol was removed from the rest of the unsaponifiable matter and the residue taken up with hot alcohol. On cooling yellowish white plates separated from the orange-coloured solution: after two or three crystallisations they were obtained as glistening white plates, M.P. 68–68·5°. The substance was a hydrocarbon containing 85·1 % C and 14·6 % H (micro-analysis). From these data the substance was identified as the hentriacontane, $C_{31}H_{64}$, originally prepared by Krafft [1882].

If green cabbage leaves are worked up similarly, the crystals separating from the hot alcoholic solution of the unsaponifiable matter melt at 72 to 75° and analysis shows that they contain about 5 % of oxygen. From these

a small amount of crystals melting at 68° was isolated, the melting point of which was unchanged when the crystals were mixed with the hentriacontane obtained from spinach. In cabbage leaves therefore the hentriacontane is accompanied by an oxygen-containing substance which is not present in spinach and which is now the subject of further investigation. The occurrence of these saturated normal hydrocarbons in green leaves is of some interest. A survey of the literature shows that three of them, containing 27, 31 and 35 carbon atoms, have been definitely identified in green leaves. These were originally prepared by Krafft [1882] by heating the barium salts of the fatty acids and reducing the resulting ketones with hydrogen iodide and phosphorus.

$$C_nH_{2n+1}.COO.Ba.OOC.C_nH_{2n+1} \rightarrow \begin{matrix} C_nH_{2n+1}.CO.C_nH_{2n+1} + BaCO_3 \\ \rightarrow C_nH_{2n+1}.CH_2.C_nH_{2n+1}. \end{matrix}$$

From myristic, palmitic and oleic (or stearic) acids, the acids most commonly occurring in plant fats, the hydrocarbons which would be produced by the above reactions are those containing respectively 27, 31 and 35 carbon atoms.

The following table shows their occurrence in plants.

$C_{27}H_{56}$ Heptacosane M.P. 59·5° C = 85·2 % H = 14·8 %	$C_{31}H_{64}$ Hentriacontane M.P. 68·5° C = 85·2 % H = 14·8 %	$C_{35}H_{72}$ Pentatriacontane M.P. 75° C = 85·3 % H = 14·7 %
	Occurrence	
	Beeswax*	
Leaves of tobacco†‡	Leaves of tobacco†‡	
	Seeds of Ko-Sam§	
	Leaves of *Gymnema sylvestre*‖	
	„ *Grindelia robusta*¶	
	„ *Morinda longiflora***	
	„ *Erodyction*††	Leaves of *Erodyction*††
	„ olive‡‡	Leaves of olive‡‡
	Bark of olive§§	
	Leaves of spinach	
	„ cabbage	

* Schwalb [1886]. † Thorpe and Holmes [1901]. ‡ Mabery [1905]. § Power and Lees [1903]. ‖ Power and Tutin [1904]. ¶ Power and Tutin [1905]. ** Barrowcliff and Tutin [1907]. †† Power and Tutin [1906]. ‡‡ Power and Tutin [1908, 1]. §§ Power and Tutin [1908, 2].

Two of the richest sources of vitamin A are found in the unsaponifiable matter obtained respectively from green leaves and from fish-liver oils and it is interesting that there is a certain parallelism between the constituents occurring in them.

Both contain (1) a highly unsaturated hydrocarbon, (2) products which may be regarded as obtained from the higher fatty acids by processes of condensation and reduction, and (3) sterols. The first of these is represented in the leaf material by the hydrocarbon carotene, containing 11 ethylenic linkages [Kuhn and Winterstein, 1928]: in the liver oil by squalene with six unsaturated linkings (or by similar unsaturated hydrocarbons). Both these hydrocarbons are built up from a number of isoprene units, and show considerable resemblance [Chapman, 1923; Heilbron, Kamm and Owens, 1926]. The second constituent is represented in the leaf by the high normal saturated

hydrocarbons enumerated above, in the liver oil by the batyl, selachyl and chimyl alcohols which are now known to be condensation products of glycerol with the higher fatty alcohols corresponding to stearic, oleic and palmitic acids [Heilbron and Owens, 1928]. Although the available evidence is against the identification of any of these substances as the active agent [Drummond, Channon and Coward, 1925], the existence of a certain similarity of composition in these two materials obtained from such widely different sources, both rich in vitamin A, is not without interest.

We desire to acknowledge our indebtedness to Dr Morgan who carried out the micro-analyses for us, and to the Department of Scientific and Industrial Research for the grants which have made the work possible.

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XVI. THE BIOCHEMISTRY OF DRY-ROT IN WOOD.

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Introduction.

The biochemical action of the saprophytic and parasitic fungi which cause decay of lignified tissues is very little understood at the present time. It is usually stated in mycological literature that the action of the fungal hyphae is to cause delignification of the lignocellulose complex by removal of the lignin and to leave a residue of cellulose. These results have been mainly attained by the study of microchemical tests on sound and decayed wood, and no systematic investigation has been made on a macroscopic scale.

Microchemical reactions applied to wood as specific tests for the presence or absence of either lignin or cellulose are of little value, as the characteristic reactions (mainly colour changes) are due to small amounts of impurities in the tissue.

It is well known that wood-destroying fungi are rich in enzymes, and as many as a dozen have been reported from a single species. The early investigations of Czapek [1899] showed that greatly increased quantities of hadromal (coniferyl aldehyde) could be extracted from wood that had been attacked by fungi, and he assumed that they contained an enzyme, hadromase, which split a supposed ether-linkage between the hadromal and cellulose. Somewhat similar results were obtained by Zeller [1916] who isolated an enzyme extract from the mycelium of *Lenzites saepiaria* which was found to act on a substrate of the sapwood of *Pinus echinata*, and an alcoholic extract of the residue gave the phloroglucinol reaction. He proposed the name lignase for Czapek's hadromase.

The investigations of Hartig [1900], based on colour changes with microchemical reagents, showed that the parasites *Polyporus vaporarius*, *P. sulphureus*, and the saprophyte *Merulius lachrymans* removed cellulose from wood and left a residue of wood-gum, while *Trametes pini* was found to remove lignin and leave pure cellulose. After the loss of lignin, the middle lamella (which was assumed to be composed of pectin) dissolved and left isolated tracheids. This latter result is obviously incorrect, as we now know that the middle lamella is composed largely of lignin.

The arguments advanced by Hartig and the majority of subsequent

investigators that decayed wood consists of pure cellulose are based upon staining the residue of this wood with zinc chloroiodide—a very unsafe standard. Other workers have also stated that members of the Polyporaceae remove lignin from wood, leaving cellulose. Lindroth [1904] found that the phloroglucinol reaction disappears first in decayed wood and then the Maule reaction also disappears. As the red colour given by wood with phloroglucinol and hydrochloric acid disappears, the colour reactions for cellulose become more intense, until finally pure cellulose is indicated.

Again, Marshall Ward [1897] by the study of colour changes with microchemical reagents came to the conclusion that the action of *Sterium hirsutum*, which attacks *Aesculus* (Chestnut), is to destroy the lignin and leave the cellulose. Ward, however, was unable to isolate any enzyme capable of causing delignification.

The evidence for the production of cellulose by *Trametes pini* is founded on stronger grounds than any of the investigations discussed above. Johnson and Lee [1923] found an increase of 15 % in cellulose and a decrease of 30 % in lignin in wood attacked by this fungus.

The first complete examination of decayed wood (Douglas Fir) was made by Rose and Lisse [1917]. Unfortunately the fungus that produced the decay was not identified. There was a marked increase in the solubility of the decayed wood in alkali and a decrease in the amount of cellulose, pentosans and acetyl groups, while the methoxyl content was increased.

From the conflict of evidence on this subject it was considered of interest to carry out an investigation of wood attacked by *Merulius lachrymans*, using modern methods of cellulose analysis. Spruce-wood (*Picea excelsa*) was chosen for the experimental work as its constants are well known and form the requisite standard of comparison for the experimental data. Only completely decayed wood was used to study the action of the fungus.

The action of *Merulius lachrymans* on timber is too well known to need detailed description. The wood loses weight and is converted into the so-called touch-wood, which powders easily to give a brown dust.

It was found that the decayed spruce-wood gave Ditz's test for oxycellulose. A small amount of the wood was suspended in water and methyl orange was added to colour the water yellow. On addition of a few cc. of a saturated solution of sodium chloride, a wine-red colour was developed at once. Sound wood did not give the reaction. A red colour was produced with phloroglucinol and hydrochloric acid. The decayed wood rapidly reduced Fehling's solution, whereas sound wood only brought about reduction after prolonged boiling. Similar results were obtained with decayed beech-wood (hard wood) which had been attacked by *M. lachrymans*. 47·2 % of the decayed wood was soluble in a 5 % solution of sodium hydroxide. The ash-content was 1·07 % and the natural moisture 13·4 %. The technique in general employed was that elaborated by Dore [1920], and in Table I a comparison is made of the values obtained for sound and decayed spruce.

Table I. *Comparison of sound and decayed spruce.*

	Sound wood %	Decayed wood %
Extracted by benzene ...	1·7	1·55
„ alcohol ...	1·6	9·46
„ water ...	2·5	5·43
„ 5 % NaOH	3·8	30·30
Cellulose	55·0	11·90
Lignin	28·0	43·00
Mannan	7·6	0·00
Galactan	0·1	0·00
	100·3	101·64
Ash-content	0·86	1·07
Natural moisture ...	10·0	13·40

(All analyses are calculated on ash-free material dried at 105°.)

An examination of the figures shows that a decrease of 60 % has occurred in the cellulose, while the lignin has increased by 57 %. It was found in the course of the analysis of the cellulose that the lignin in the wood had in some way been altered. When the wood was placed in chlorine gas to chlorinate the lignin prior to its removal by boiling with sodium sulphite solution, instead of the usual golden-yellow colour being formed, the whole mass turned a dull brick-red. A further point in this connection is the complete removal of the hexosans, mannan and galactan. The portion soluble in 5 % sodium hydroxide solution (after removal of the fractions soluble in benzene, alcohol and water respectively[1]) represents, in part at any rate, the hemicellulose components of the wood. These bodies are soluble in dilute caustic alkali and are readily hydrolysed by acids. They contain "uronic" acids of the type of galacturonic acid [Nanji, Paton and Ling, 1925], as well as pentose and hexose residues. It has been shown by O'Dwyer [1926] that the hemicellulose fraction of beech-wood consists of two substances, hemicellulose A, which is precipitated from alkaline solution by acid, and hemicellulose B, soluble in water but insoluble in alcohol. Hemicellulose A contains xylose and 11 % of glycuronic acid, whereas hemicellulose B contains arabinose and 63 % of galacturonic acid as well as small amounts of galactose. Dorée and Barton-Wright [1927] have also shown that two hemicelluloses are present in spruce-wood. That a part at least of the sodium hydroxide-soluble fraction of decayed wood consists of hemi-celluloses was indicated by the presence of increased amounts of uronic acids. These on distillation with 12 % hydrochloric acid do not give a quantitative yield of furfural; but the carbon dioxide evolved by the decarboxylation of their carboxyl group affords an exact measure of their amount. The yield of uronic acid from the good wood was 2·688 %, and from the decayed wood 4·84 %; an increase of 82 %. The greater part of the alkali-soluble fraction was precipitated by the addition of acetic acid, and the remainder, after con-centration, by adding alcohol [cf. O'Dwyer, 1926]. The increase in solubility of the decayed wood in NaOH solution compared with that of sound spruce

[1] The total of the values found for the benzene, alcohol, water and 5 % sodium hydroxide extract (47·2 %) agrees well with the solubility of the wood in 5 % sodium hydroxide alone (46·71 %), without preliminary extraction with organic solvents and water.

is very high and cannot be entirely accounted for by the presence of hemicelluloses. It is possible that the remainder of the alkali-soluble fraction is composed of the metabolic products of the fungus and the contents of the fungal hyphae.

Comparison of the figures for the methoxyl content of sound and decayed wood shows an increase in the latter of 77 % (sound wood, OMe = 5·44 %; decayed wood, OMe = 9·67 %). A small part of the methoxyl content is located in the hemicellulose fraction [see O'Dwyer, 1928], while the rest occurs in the lignin.

We have obtained evidence (which we hope to publish in a later communication) that the major part of the alcohol extract is composed of lignin. It is possible that it is of the nature of the metalignin isolated by Dorée and Barton-Wright [1927], which has the molecular composition $C_{20}H_{20}O_6$ and is the simplest type of lignin known.

Discussion.

Direct determination of the cellulose and lignin in decayed wood shows decrease of the former and increase of the latter, and estimation of the methoxyl groupings confirms the increase of the lignin. These results show very clearly that no delignification of the wood is brought about by the fungus, but rather that the main attack is confined to the cellulose. The hexosans, mannan and galactan, are also removed, and we would therefore suggest that *M. lachrymans* first attacks these two easily hydrolysed bodies in the course of its metabolic activities and then confines its attack to the cellulose.

It is difficult to account for the increase in the hemicellulose fraction of the decayed wood. The hemicelluloses are readily hydrolysed substances, and the main hydrolytic products are sugars. On the other hand uronic acids are also produced, and it may well be due to the presence of the latter that the fungus does not affect the hemicelluloses.

The influence of the hydrogen ion concentration of the medium on the development of the mycelia of wood-destroying fungi has only been examined in a few cases, but Meacham [1918] found that the first marked deflection in the growth curve occurred at an acidity of $N/350$ HCl and was limited by $N/50$. The hydrogen ion concentration, however, does not appear to be the chief factor determining a desirable medium. According to Zeller, Schmitz and Duggar [1919], no general statement can be made on the relationship between the p_H of the medium and the growth of wood-destroying fungi.

Merulius lachrymans is very sensitive to the presence of acids. Wehmer [1911, 1912] has found that both gallic and tannic acids are markedly toxic to the fungus, and it is for this reason that coniferous (soft) wood is very much more rapidly attacked than a hard wood such as oak. Concentrations of 1–2 % of these acids at once stop all mycelial growth, and coniferous wood can be made resistant to *Merulius* by impregnation with tannic acid. The presence of acids produced by hydrolysis would seriously hamper the fungus in its

attack on the wood, and it may be on this account that the hemicelluloses are not affected. On the other hand, the hydrolysis of the galactan, mannan and cellulose fractions leads to the production of no growth-inhibiting products, but provides the requisite carbohydrate material for active metabolism. With the destruction of the cellulose matrix of the tracheids the touch-wood or tinder-wood condition, which is so typical of wood affected by *M. lachrymans*, is obtained.

SUMMARY.

The effect of the fungus *Merulius lachrymans* (the cause of dry-rot in wood) is to remove the galactan, mannan and cellulose fractions in spruce-wood; the hemicelluloses and lignin are not affected, *i.e.* no delignification of the woody tissues takes place.

We are indebted to Mr S. O. S. Dark for the uronic acid estimations, and to Mr C. S. Semmens for his invaluable assistance in a number of ways.

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XVII. REDUCTION POTENTIAL, ENERGY EXCHANGE AND CELL GROWTH.

EXPERIMENTS WITH *B. COLI*.

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PART I.

Introduction.

So far biological interest in oxidation-reduction potentials has centred mainly on the determination and significance of intracellular r_H. The experimental method has been to inject into cells oxidation-reduction indicators (whose r_H ranges have been determined by Mansfield Clark and his colleagues) and to observe the equilibria obtained between their reduced and oxidised forms.

Needham and Needham [1926] came to the conclusion that "the r_H of the amoeba is probably widely independent of the concentration of oxygen in the external atmosphere" but that an anaerobic protozoon (*Nyctotherus cordiformis*) has "an internal r_H of 19 to 20 under aerobic conditions whilst under anaerobic conditions the latter value changes to r_H 9·5 to 10·5." They considered that "in the facultative anaerobe the oxidation-reduction potential can be adjusted to the environment whilst in the strict aerobe changes in the environment which would lead to such an alteration in the r_H bring about death." Their conclusion that the cell has "a definite, well poised and characteristic equilibrium" was based essentially upon the fact that certain oxidation-reduction indicators became oxidised when injected under anaerobic conditions into the cell in their reduced forms.

Rapkine and Wurmser [1927] have determined the r_H of a number of cells and found them all to be in the zone 19–20. This value was not altered by the injection into the cell of such substances as glucose, sodium pyruvate and succinate. They concluded that "the rate of dehydrogenation is, within wide limits, independent of the concentration of hydrogen donators."

Recently Cohen, Chambers and Reznikoff [1928] have injected twenty-five oxidation-reduction indicators in either oxidised or reduced form into *Amœba dubia* and *Amœba proteus* under controlled oxygen access. They found, contrary to the Needhams, that under anaerobic conditions the amœba was able to reduce *all* the indicators used and that it could not oxidise six of the most easily oxidisable indicators. The phenomena were closely parallel to those observed by Cannan, Cohen and Clark [1926] in their reduction electrode studies on bacterial cultures.

At present, therefore, there is evidence by the Needhams pointing to the existence of a fairly well poised oxidation-reduction system in the cell and there is the opposing evidence of the American workers indicating the existence of an intracellular reduction intensity which increases as the neutralising effect of oxygen is eliminated.

It is difficult to estimate, however, how far r_H terminology can be applied to the living cell. The application of r_H terminology implicitly assumes the existence in the cell of equilibria arrived at almost instantaneously in a homogeneous reversible system. Yet the cell is a heterogeneous system; its enzymes and catalysts are in a heterogeneous condition. From the work of Brooks [1926], Rapkine and Wurmser [1926] and Cannan [1926] it is known, for example, that relatively high reducing potentials can be maintained in the

green cell apparently in the presence of free or molecular oxygen. It is doubtful therefore what significance can be given to the actual value of intracellular r_H; its value (determined by the ratio of reduced to oxidised form of indicator) is a function of many factors, inevitable in a heterogeneous system of catalysts, and to perceive its significance it is necessary to disentangle and estimate the influence of each factor.

Michaelis and Flexner [1928] point out that when a tissue, naturally aerobic, is kept under anaerobic conditions it undergoes reactions which ultimately reach an equilibrium, the final potential obtained being in some way related to the chemical composition of the tissue in its living state. But, granting that such a final equilibrium potential can be determined, it is difficult to see how it can be interpreted to give some idea of the state of the cell under normal conditions; for it is with velocities of change that the normal events of the cell are primarily concerned and these velocities may in many cases be independent of the magnitude of a potential.

We have approached the problem of the relationship of r_H to events in the cell in a different manner from that which has hitherto been adopted.

Quastel and Stephenson [1926] suggested that the facts relating to the action of oxygen on the proliferation of a strict anaerobe such as *B. sporogenes* could be best interpreted by assuming that the cell must attain a certain limiting reduction potential before proliferation could occur. At a potential more oxidising than this the cell would fail to proliferate; below this potential proliferation was possible. This point of view has been confirmed by the recent work of Aubel, Aubertin and Genevois [1928].

The question arose as to whether a facultative anaerobe such as *B. coli* needed a definite r_H range within which to proliferate and this led to the enquiry: does the growth of the cell depend at all on the oxidising or reducing intensity, on the r_H, of the environment? The experiments on *B. sporogenes* seem to show that a particular value of reduction potential is necessary before proliferation of the anaerobe can occur. But is this phenomenon a general one? The following communication constitutes an attempt to answer this question.

Experimental method.

Our experimental method, briefly, has been to determine how far the growth of *B. coli* on synthetic media is affected by changes in the reducing or oxidising power of the media in presence of the cell. The work has been made possible by a knowledge of the reactions brought about by the various constituents of the media in presence of the "resting" or non-proliferating organism.

Throughout the experiments described below, the inorganic medium employed was as follows:

0·4 g. $(NH_4)_2HPO_4$.	0·07 g. $MgSO_4$, $7H_2O$.
0·1 g. NaCl.	Trace $FeSO_4$.
0·1 g. KH_2PO_4.	100 cc. distilled water.

This inorganic medium was usually made up to three times the strength given and kept as stock. When made up into a nutritional medium it was always so diluted that the final strength of salts was that given. The organic acids contained in the media were added in the form of their sodium salts and the final p_H of all media was 7·2. All experiments were done in duplicate, and series of experiments were repeated many times. 5 cc. of each medium, after autoclaving, were sown with 2 drops of a freshly grown tryptic broth culture of *B. coli*. Usually 20-hour growths were used but 6-hour growths are very satisfactory. It is advisable to use young cultures, results with old ones being inconsistent and variable. A heavy sowing, such as that given in 2 drops, was usually found to be necessary. A number of strains of *B. coli* have been used. It is preferable for consistent results to use a strain which gives a good growth, in tryptic broth at 37°, within 6 hours.

In certain experiments, especially when using slowly growing strains of *B. coli*, a trace of an alcoholic extract of marmite was added to the media, controls always being carried out to show that no perceptible growth occurred on the quantity of marmite alone.

Growths were usually examined after 20–40 hours' incubation (aerobic and anaerobic) at 37°. Details of the composition of the various media are given in the tables which summarise the results.

Figures are given in the tables which express relative growths.

The growths were usually estimated by comparison with standard barium sulphate suspensions or nephelometrically. In a number of cases bacterial counts were made but such absolute values were not so important for our purposes as the relative ones. The figures in one table representing relative growths cannot be compared with the figures given in another table, the tabulation being such that the results given in any one particular table can be strictly compared with each other but not with those in any other table.

Synthetic media for anaerobic growth of *B. coli*.

The following three conditions, at least, appear to be necessary before proliferation can occur.

1. The organism must be able to secure energy either by anaerobic decomposition of the substrate (*e.g.* glucose into lactic acid) or by the oxidation of the substrate.

2. The organism must be able to activate the substrate so that the latter is capable of reaction, *e.g.* oxidation or reduction (which it does not necessarily undergo in absence of the cell).

3. The products of oxidation (or decomposition) of the substrate, or the substrate itself, must be capable of entering into the synthetic operations of the organism.

It follows that if molecular oxygen be replaced by some molecule which induces in or around the bacterial cell a state comparable with that which occurs under aerobic conditions and so long as the three conditions stated

above are fulfilled anaerobic growth should occur in presence of this molecule. Now *B. coli* activates nitrates and fumarates as hydrogen acceptors and the conditions enumerated above hold in the systems lactate-nitrate and lactate-fumarate. Anaerobic growth of *B. coli* was found to occur in both media, the lactate giving rise on oxidation to pyruvate which was utilised by the organism [Quastel, Stephenson and Whetham, 1925; Quastel, 1925]. Later it was shown that anaerobic growth occurred, as was anticipated, on glycerol-nitrate, glycerol-fumarate and glycerol-aspartate media and that it did not occur with a strict aerobe, such as *B. alkaligenes* which failed to show any perceptible activating action on nitrates or fumarates [Quastel and Stephenson, 1925; Quastel and Wooldridge, 1925].

In a lactate-fumarate medium, the lactate acts, in presence of the cell, as a hydrogen donator and must be regarded therefore as exerting a reduction potential relative to the cell. Similarly the fumarate must be considered as exerting, in presence of the cell, an oxidation potential. Therefore, although a lactate-fumarate medium, in the absence of the cell, has no observable reducing or oxidising power[1] and gives no potential discoverable at an electrode, it must be considered as exerting an oxidation-reduction potential, dependent on the relative concentrations of the two substances, in presence of the cell. This potential is not observable at an electrode because of the inaccessibility at the electrode of the cell enzymes in combination with their substrates—nevertheless it is clearly indicated by the fact that methylene blue, in presence of the system (lactate-fumarate), will be reduced to a particular value dependent on the relative velocities of reduction by lactate and oxidation by fumarate. Such a value would be as real an indication of the r_H of the medium relative to the cell, as is the intracellular r_H of a cell indicated by the method of microinjection. Now if the growth of *B. coli* is dependent on the r_H of the medium it should be dependent on the relative concentrations of lactate and fumarate in the medium.

ANAEROBIC GROWTH OF *B. COLI* ON A LACTATE-FUMARATE MEDIUM (TABLE I).

Table I.

Growth of *B. coli* anaerobically on media containing lactate and fumarate in varying concentrations. Each column represents a separate medium. Growths examined nephelometrically.

Conc. of lactate × $M/10$	1	1	1	1	1	1	1	1·5	2	3	4	4
Conc. of fumarate × $M/40$	0	0·5	1	1·5	2	3	4	1	1	1	1	2
Relative anaerobic growths after 20 hrs. incubation	0	0·5	1·5	2	2	2	1·5	2·5	1·5	0·5	0	0

The following conclusions may be drawn.

1. Growth increases with increase of lactate concentration until a concentration is reached above which growth diminishes with increase in concentration.

2. Growth increases with fumarate concentration until a maximum is reached. This concentration is about $M/40$. Above this concentration further

[1] On, say, the methylene blue system.

increase of fumarate appears to have but little inhibitory action. High concentrations of fumarate are however definitely inhibitory.

These results are in accordance with those found by Quastel and Stephenson [1925].

The fact that growth increases with increase in lactate and fumarate concentration until maxima in concentrations are reached is easily understood, for the rate of growth must be dependent on the rate of oxidation of the lactate to pyruvate (which is assimilated by the organism) and upon the rate of energy liberation (which is used for synthetic purposes). Such rates must be dependent on the amounts of lactate and fumarate present.

The inhibitory action of lactate at relatively high concentration is not due to any toxic effect of this substance on *B. coli*, for good aerobic growth will occur in lactate-fumarate media where the concentrations of lactate are such that anaerobic growth is markedly inhibited.

The inhibition of growth with increase of lactate concentration may be due, however, to an increased reduction potential being secured within the cell. If this be so it will be expected that increase in fumarate concentration, after attaining a critical value, will balance the inhibitory action of increased concentration of lactate and so result in an increased growth. This does not seem to occur. It may well be argued, however, that the failure of fumarate to balance the lactate effect is due to saturation of the fumarate enzyme with its substrate, so that increase in concentration of the latter does not exert any increased velocity of oxidation and hence any power of neutralising the lactate effect.

This difficulty may be surmounted in the following manner.

In the presence of *B. coli* an equilibrium is established between succinate, fumarate, methylene blue and leucomethylene blue, the equilibrium point being independent of the absolute quantities of succinate and fumarate present [Quastel and Whetham, 1924]. The oxidising action of fumarate is balanced by the reducing action of succinate in a reversible manner characteristic of the usual reduction-oxidation indicators.

If now to a lactate-fumarate medium succinate be added, the r_H of the medium (relative to the cell) must decrease, the fumarate action being opposed by that of the succinate. The evidence points to the same enzyme activating both succinate and fumarate [Quastel and Wooldridge, 1927, 1] so that the difficulty of enzyme saturation does not appear here. Clearly, whatever the concentrations of the two substances, their ratio will determine the reduction potential at their enzyme, *i.e.* in the presence of the cell. Hence by keeping the lactate concentration constant the r_H may be varied by varying the relative proportions of succinate to fumarate.

Anaerobic growth of *B. coli* on a lactate-fumarate-succinate medium.

The results are shown in Table II.

1. Increase in succinate concentration results in a marked inhibition of growth.

2. This inhibition is relatively greater than that produced by increase in lactate concentration.

Table II.

Growth of *B. coli* anaerobically on lactate-fumarate-succinate media of varying compositions.

Conc. of lactate × *M*/10	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5	0·5
Conc. of fumarate × *M*/40	0·5	0·5	0·5	0·5	0·5	0·5	1	1	1	1	1	1	2	2	2	2	2	2	2
Conc. of succinate × *M*/40	—	0·25	0·5	1	1·5	2	—	0·25	0·5	1	1·5	2	—	0·25	0·5	1	1·5	2	3
Relative anaerobic growths after 20 hours' incubation	2	2	1·7	1·3	1	0·5	3	3	2·2	1·9	1·4	0·5	3	2·9	1·7	1·1	0·8	0·75	0·5

The specific action of succinate.

The inhibiting action of succinate may be shown to be specifically connected with fumarate. Anaerobic growth of *B. coli* in tryptic broth, glucose solution, sodium pyruvate solution or a lactate-nitrate medium is not perceptibly inhibited by the presence of concentrations of succinate which markedly inhibit growth in a lactate-fumarate medium (see Table III). This specific action of succinates supports the view that the oxidising action of fumarate is being opposed by a reducing action of the succinate. It may be shown that the inhibition of growth is not due to toxicity of succinate, in this particular

Table III.

Growth of *B. coli* anaerobically in various media in the presence and absence of succinate. Growths examined after 20 hours' incubation.

	Tryptic broth	Lactate-fumarate	Lactate-nitrate	Glucose *M*/20	Pyruvate *M*/10
Without succinate added	2	2	2	2	2
In the presence of succinate *M*/10	2	0·5	1·5	2	2

medium, for if nitrate be added to such a growth-inhibitive medium, an accelerated growth results. Again a lactate-fumarate-succinate medium exposed aerobically gives a good growth. The effect of the succinate is not due to osmotic action; the same molar concentration of NaCl has no effect on a lactate-fumarate medium. These results are noted in Tables IV and V.

Table IV.

To show anaerobic growths of *B. coli* in lactate-fumarate-succinate media accelerated by the presence of nitrates.

Conc. of lactate × *M*/20	1	1	1	1	1	1
Conc. of fumarate × *M*/40	1·5	1·5	1·5	3	3	3
Conc. of succinate × *M*/40	2	2	2	2	2	2
Conc. of nitrate × *M*/10	—	0·5	1	—	0·5	1
Relative anaerobic growth after 20 hrs. incubation	0·6	2	2	0·4	2	2

The effects of substances other than succinate on the anaerobic growth of *B. coli*.

So far the evidence would appear to favour the conclusion that the anaerobic growth of *B. coli* in a lactate-fumarate medium diminishes with increased concentration of succinate because the latter is responsible for an increase in reduction potential. The results do not show very clearly, however, that growth is dependent on any ratio of succinate to fumarate. There is remarkably little indication, on the whole, that increase of fumarate concentration neutralises the inhibitive action of increased succinate concentration.

Another explanation of the phenomenon is possible. The same effect of succinate on the anaerobic growth of *B. coli* in a lactate-fumarate medium would obtain if succinates were adsorbed at the fumarate enzyme so that the amount of active fumarate available for hydrogen acceptance would be reduced. Thus if the addition of succinate simply had the effect of competing with fumarate for its enzyme, there would result a diminution in the velocity of oxidation of the lactate. This would not only result in a diminution in the rate of liberation of energy but in a diminution in the rate of production of pyruvate necessary for assimilation. It is easy to see therefore how the presence of succinate would retard cell growth. If this view is true substances other than succinate, perhaps without any reducing action, would bring about the same effect—so long as they competed with either fumarate or lactate for their respective enzymes.

It is known from results on resting bacteria [Quastel and Wooldridge, 1928] that the following substances are among those which compete with lactate for its enzyme: glycollate, α-hydroxybutyrate, oxalate, hydroxymalonate. Malonate competes with succinate for its enzyme. None of these substances is comparable with succinate or lactate for reducing activity at relatively low concentrations; oxalate and malonate have no perceptible reducing action in presence of *B. coli*. Table V shows the effects of these substances on the anaerobic growth of *B. coli* in a lactate-fumarate medium. It will be seen that the substances which compete with lactate or fumarate for their enzymes inhibit growth. The same molar concentration of NaCl has no effect. The same molar concentration of acetate, which does not compete markedly with either lactate or fumarate, has relatively little effect[1].

The evidence points therefore to the action of succinate being just as much due to its competition with fumarate as to its exerting a reduction potential.

This is borne out by another experiment. As indicated earlier *B. coli* will grow anaerobically in a glycerol-fumarate medium. The growth in such a medium takes place with much lower concentrations of fumarate than will allow anaerobic growth in a lactate medium, *i.e.* a smaller number of activated fumarate molecules are necessary for growth on glycerol than for growth on

[1] Significance should not be attached to the results obtained on the addition of acetate to nitrate media since growth of *B. coli* occurs anaerobically in an acetate-nitrate medium.

lactate. (An explanation for this will be suggested later.) It would be possible, if the competition theory were true, that the addition of succinate to a glycerol-fumarate medium, whilst diminishing the total number of activated fumarate molecules, would still leave sufficient of the latter to induce relatively unaltered growth. Experiment (Table V) shows that a concentration of succinate which markedly inhibits growth in a lactate-fumarate medium has but little or no effect in a glycerol-fumarate medium, the amounts of fumarate being in each medium the same.

Table V.

To show the effect of the addition of various substrates to basal media, containing a hydrogen donator and acceptor, upon the anaerobic growth of *B. coli*. Each tube contains 5 cc. of medium consisting of the specified inorganic medium together with the concentrations of H-donator and H-acceptor given in the first column. The substrates are noted at the head of subsequent columns; each being present in *M*/20 concentration. Two drops of an 18-hour broth culture of *B. coli* were inoculated into each tube. The resulting growth after 30 hours' incubation at 37° anaerobically was estimated nephelometrically and is given by the figures in the table. The growths of each series are mutually comparable. ? = doubtful growth.

	Control	NaCl	Acetate	Oxalate	Hydroxy-butyrate	Gly-collate	Hydroxy-malo-nate	Malo-nate	Succinate
M/10 lactate* *M*/20 fumarate	3	3	2	1	0·25	0·25	1	1	0·5
M/100 lactate* *M*/100 nitrate	2	2	2	1·5	1·5	1·5	1·5	0·5	1·5
M/100 fumarate* *M*/100 nitrate	2	2	2	1·5	1·5	2	2	?	1
M/20 acetate* *M*/20 nitrate	1·5	2	1·5	2	1·5	2	3	0·75	2
M/50 glycerol *M*/50 fumarate	2	3	2	4	2	1	3	3	3
M/50 glycerol *M*/50 nitrate	3	3	3	4	2·5	2·5	4	1·5	3
M/20 glucose	2	2	1	2	2	2	1·5	2	2
M/10 pyruvate	2	2	1	2	2	2	3	3	3

* These series contain 0·5 cc. of alcoholic extract of marmite (1/250), control tubes showing no perceptible growth.

Competitive action.

The specific succinate effect seems to be parallel to the effect of carbon monoxide in competing with oxygen for the oxidase system [Warburg, 1926; Keilin, 1927]. The reversibility exhibited by the CO-O_2-oxidase system, is not so easily demonstrable, however, in the succinate-fumarate system. At most we have only had indications that the inhibitive action of succinate is balanced by an increased concentration of fumarate. Further experiment is still needed to establish this finally. The experimental difficulty is that high concentrations of salts undoubtedly inhibit growth by their osmotic effect; so that only a restricted range of concentrations is possible within which to test for reversibility and the range may not be great enough for the purpose. Very low concentrations of fumarate cannot be used for experiment because at these concentrations a small increase of fumarate will, in the absence of succinate, bring about an appreciably increased rate of growth.

The competitions between succinate and malonate and between lactate and oxalate at their respective enzymes are reversible [Quastel and Wooldridge, 1928], the inhibition of reduction by a competing molecule such as malonate or oxalate not being directly proportional to the concentration of the latter but taking place at a rate which would be anticipated if adsorption occurred. Eadie [1928], who has confirmed the observation [Quastel and Whetham, 1925] that the velocity of reduction of methylene blue by a donator in presence of *B. coli* follows the logarithmic relationship

$$v = k + k' \log C,$$

(where k, k' are constants, C is concentration and v is velocity), concludes that this relationship is best interpreted on the basis of adsorption of the substrate at its enzyme.

It would seem a reasonable argument, in face of the evidence, that the succinate effect is due more to competitive adsorption at the fumarate enzyme than to the succinate exerting a reduction potential effect. The matter has been put to further test by investigating the action of succinates and other substances on a variety of media capable of supporting anaerobic growth. Table V summarises the results. It will be observed that inhibition of growth occurs where direct competition of the additional substance with a substrate of the medium is known to occur [Quastel and Wooldridge, 1928].

Conclusions on the action of succinate.

Part, at any rate, of the inhibiting action of succinate may be attributed to its competing with fumarate for its enzyme. There is left the possibility that some of the action is due to an increased reduction potential. It may be considered that the competition itself is synonymous with the production of an increased reduction potential owing to the diminution in number of activated fumarate molecules and hence in the velocity of oxidation. The intracellular r_H would indeed change to a more negative (reducing) value if the velocity of oxidation by fumarate within the cell were to decrease—assuming no marked poising action within the cell. But the same effect would occur by competition with the lactate molecules; here, in contrast to fumarate, the intracellular r_H would become more positive owing to a diminution in the velocity of reduction. In both cases a diminution of growth occurs. It may appear that there is just one value of intracellular r_H at which the rate of proliferation is greatest; that it diminishes as the r_H becomes more oxidising. But in presence of oxygen when presumably the r_H is still more positive the rate of growth is increased. Hence it becomes difficult, if not impossible, to relate the growth of the cell directly to its internal r_H.

PART II.

It is desirable now to consider the effects of the addition to the basal medium of a substance, normal to tissues, but independent of the cell or its enzymes for its reducing action. Such a substance is glutathione and the experiments to be described show the action of glutathione, cysteine and thioglycollate on the growth of *B. coli*. Details of the experimental technique will be found in Part I.

Effects of cysteine, glutathione and thioglycollate on the growth of *B. coli* on a lactate-fumarate medium.

(A) *Anaerobic* (see Table VI).

It will be seen that these —SH compounds[1] affect the rate of anaerobic growth markedly. There is a considerable diminution in the growths, these results being clearly observed after 30–40 hours' anaerobic incubation.

(B) *Aerobic* (see Table VI).

The growths under aerobic conditions are, on the other hand, after 20–40 hours' incubation, at least as profuse as, and often greater than, the growths in the media containing no —SH.

Table VI.

To show effects of —SH compounds on aerobic and anaerobic growth of *B. coli* on lactate-fumarate media at p_H 7·2. Growths were examined nephelometrically after 40 hours' incubation. The —SH compounds were sterilised separately by steaming.

Conc. of lactate × *M*/20 ...	1	1	1	1	1	1	1
Conc. of fumarate × *M*/40 ...	1	1	1	1	1	1	1
Conc. of cysteine × *M*/156 ...	—	0·5	1	—	—	—	—
Conc. of glutathione × *M*/250	—	—	—	0·5	1	—	—
Conc. of thioglycollate × *M*/92	—	—	—	—	—	0·1	0·2
Relative growths aerobic	2·5	5	3	4	4	2·5	3·5
Relative growths anaerobic	2	0·7	0·5	0·8	0·6	1·2	0·5

To account for this phenomenon it might be suggested that under aerobic conditions all the —SH is oxidised by molecular oxygen, so that there is no —SH left to give a high reduction potential. The maintenance in the cell of this potential might account for the diminution under anaerobic conditions in the rate of proliferation of the organism.

Quantity of —SH present after growth under aerobic and anaerobic conditions.

To test this view the amounts of —SH left in the medium after growth under aerobic and anaerobic conditions were estimated by titration with dilute iodine solution (Table VII).

In almost every case it was found that the amount of —SH left in the medium under aerobic conditions was equal to, and often greater than, the amount left under anaerobic conditions. Thus, in spite of the fact that there

[1] It is very convenient to speak of —SH as though it were a separate entity.

was present in the medium sufficient —SH to give a high negative reduction potential [Dixon and Quastel, 1923; Michaelis and Flexner, 1928], aerobic growth proceeded apparently unaffected whilst anaerobic growth was markedly inhibited.

Table VII.

To show quantities of —SH (estimated by titration with $N/200$ iodine solution) present in lactate-fumarate —SH media after 40 hours' incubation under aerobic and anaerobic conditions.

Lactate-fumarate media	Relative growths anaerobic	cc. $N/200$ iodine required for 5 cc. medium	Relative growths aerobic	cc. $N/200$ iodine required for 5 cc. medium
Exp. 1.				
Without —SH	2·6	0·1	3·7	0·1
With cysteine initial conc. $M/312$ ...	1·0	1·5	5·5	1·6
With glutathione initial conc. $M/250$	1·2	2·2	5·7	1·6
Exp. 2.				
Without —SH	2·0	0·1	2·5	0·1
With cysteine initial conc. $M/156$ ...	0·5	0·85	3·0	1·15
With glutathione initial conc. $M/250$	0·6	1·4	4·0	1·65

The fact that aerobic growth obtains under such conditions is reminiscent of the work of Brooks, Rapkine and Wurmser, and Cannan, which shows that high reduction potentials can occur within the green cell apparently in the presence of free or molecular oxygen. The fact, too, reminds us of the experiments of Hopkins [1925] on the oxidation of protein in presence of glutathione. Throughout the oxidation, even when the velocity of the latter was at its greatest, a small but definite concentration of —SH was maintained. As Hopkins said, the maintenance of this concentration was determined by the relative velocities of reduction of —S.S— by the protein and of oxidation of —SH by oxygen.

Effects of nitrates and oxygen.

If to a lactate-fumarate medium containing —SH, which therefore only feebly supports the anaerobic growth of *B. coli*, there be added nitrates, an accelerated growth of the organism is observed (Table VIII).

Table VIII.

To show the effects of nitrate on anaerobic growth of *B. coli* in lactate-fumarate —SH media

Conc. of lactate × $M/20$	1	1	1	1	1
Conc. of fumarate × $M/40$	1	1	1	1	1
Conc. of cysteine × $M/156$	—	0·5	1	0·5	1
Conc. of nitrate × $M/1·7$	—	—	—	0·5	0·5
Relative growths after 40 hours' anaerobic incubation	2·0	0·7	0·5	2·1	1·9

In the case of aerobic growth on the —SH media, there is usually a lag in the initial rate of growth which is dependent upon the amount of —SH initially introduced into the medium. But the lag disappears after 30 hours' or less incubation and then there results a very profuse growth. This lag we attribute to the medium being essentially anaerobic in the presence of excess

of —SH; as soon as the excess is removed by oxidation, molecular oxygen becomes freely available to the cells and aerobic growth proper commences.

The quantity of —SH measured after 30–40 hours' incubation does not diminish after further incubation either aerobically or anaerobically; rather does it tend to increase.

The aerobic growth of *B. coli* on the —SH media is not due simply to a growth on the —SH compounds introduced. Control experiments show that the growth on these substances in the concentrations used is small compared with the growths obtained in the lactate-fumarate —SH media. Thioglycollic acid[1], upon which the growth of *B. coli* is most feeble, has similar effects, aerobically and anaerobically, to glutathione.

Interpretation of the effects of —SH.

The fact that aerobic growth is unaffected and anaerobic growth is greatly diminished by the presence of —SH in a lactate-fumarate medium, and that in both cases the same amount of —SH is maintained, points to the conclusion that the ability of the cell to proliferate cannot be directly related to the reduction potential of the medium—if by reduction potential is meant the potential secured by the electromotively active substance present.

Now it has been shown by Kendall and Nord [1926] and by Michaelis and Flexner [1928] that the potential of cysteine at an electrode is markedly affected by traces of free oxygen. What, then, are we to consider the potential of the media which we have employed?

It is difficult to apply r_H terminology to such electromotively unbalanced systems as these. It can be argued that the —SH present in the medium maintains within the cell a reduction potential which becomes more positive in the presence of oxygen than in the presence of fumarate. Proliferation presumably would only occur normally at the more positive value. But if proliferation depends essentially on the maintenance of this more positive potential, why should anaerobic growth of *B. coli* occur on glucose? Here reducing conditions are so intense that, as is well known, hydrogen is evolved. Consideration of potential alone seems to give no satisfactory interpretation. This seems to be inevitable, for the rate of growth necessarily depends upon velocity relations, and only in so far as reduction potentials affect relative velocities can we expect to connect rates of growth with potential measurements.

The question of intracellular hydrogen carriers.

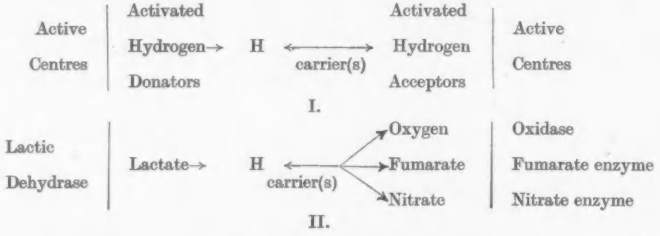
When *B. coli* grows anaerobically on a lactate-fumarate medium, by what means is the hydrogen transferred from the lactate to the fumarate? The two enzymes governing the activation of lactate and fumarate appear from the work on resting bacteria to be distinct from each other[2].

[1] To perceive the effects of these —SH compounds only relatively small quantities must be used.

[2] Some "overlapping" occurs in the case of the lactic and succinic enzymes when these enzymes are studied with normal *B. coli* [Quastel and Wooldridge, 1927, 2].

The enquiry resolves itself into the following problem: will a hydrogen donator react with a hydrogen acceptor in the presence of their specific enzymes but in the absence of any intermediate hydrogen carrier?

Now if such a reaction could occur in the absence of a carrier we would expect that a hydrogen donator, or acceptor, in the presence of its enzyme would be electromotively active, *i.e.* it would give a potential at an electrode in a manner similar to —SH or methylene blue. The experiments bearing on this point do not, however, indicate that a hydrogen donator in the presence of its enzyme has a behaviour characteristic of an electromotively active system. Indeed the experiments of Kodama [1926] on hypoxanthine in presence of xanthine oxidase show that no such potential is produced. Keilin has been able to demonstrate that cytochrome plays the part of hydrogen carrier between oxygen activated by oxidase and hydrogen donators activated by dehydrases. It seems equally possible that in the cell of *B. coli* a carrier transfers hydrogen from the activated donators to the activated acceptors. It remains for experiment[1] finally to decide this, but in the meantime we feel justified in proceeding on the assumption that such intracellular hydrogen carriers are necessary. The scheme is represented in diagrams I and II.



The carrier[2] is presumably of the usual oxidation-reduction type. Let its oxidised form be represented by A and its reduced form by AH_2. Then since it is diffusible in the cell (otherwise hydrogen carriage would be impossible) the level of oxidation-reduction potential in the cell will be determined by the ratio $[A]/[AH_2]$. This ratio will be determined by the relative velocities of the reducing and oxidising processes within the cell. Now the rate of oxidation of a hydrogen donator, say lactate, will be determined by the concentration of A and the rate of reduction of a hydrogen acceptor, say fumarate, by the concentration of AH_2. The rates will, of course, be dependent also on the concentrations of donator and acceptor but we may assume that the cell enzymes are saturated with regard to their substrates, so that variation of substrate concentration above their saturation points will not affect velocities.

The growth of *B. coli* will be dependent on at least two factors:

(*a*) the rate of production of pyruvate from lactate,

(*b*) the rate of liberation of energy by oxidation.

[1] Such experiments are now being carried out.

[2] There may be a number of carriers but this does not affect the discussion.

These rates will be determined by the value [A] and hence by the oxidising and reducing processes present. If the value [A] be diminished by the introduction into a cell of another reducing factor, say —SH, the proliferation of the cell will be affected through changes in the rates of pyruvate and energy production. If the rate of reduction of A by —SH is greater than the rate of oxidation of AH_2 by the acceptor, the value [A] will decrease with a consequent decrease in the rate of proliferation.

This, we suggest, is a possible explanation of the effects of —SH on the aerobic and anaerobic growth of *B. coli* on a lactate-fumarate medium. It is known from work on the resting organism that the rate of oxidation of leucomethylene blue by oxygen is much greater than by fumarate. Hence we may expect the rate of oxidation of AH_2 to be greater with oxygen than with fumarate. If therefore the amount of —SH in the cell remains constant, the value of [A] will be greater in the case of oxygen than in the case of fumarate. This value may be sufficiently large in the case of oxygen[1] to allow the proliferation of the cell to proceed unaffected, but in the case of fumarate too small for normal growth.

Energy requirements of the cell.

The rate of growth, as has been stated, depends on at least two rates, those of production of pyruvate and of energy. Now it is possible that there may be two molecules, each capable of giving rise to pyruvate, but whose energy productions per molecule may differ from each other considerably. An example of a pair of such molecules is glycerol and lactate. The yield of energy per molecule in breaking down to pyruvic acid is shown in the following equations:

$$CH_3.CH(OH).COOH + COOH.CH{:}CH.COOH = CH_3.CO.COOH + COOH.CH_2.CH_2.COOH + 14 \text{ Cal.}$$

$$CH_2(OH).CH(OH).CH_2OH + 2COOH.CH{:}CH.COOH = CH_3.CO.COOH + 2COOH.CH_2.CH_2.COOH + 50 \text{ Cal.}$$

It is easily seen that if the rate of oxidation of glycerol is small compared with that of lactate the rate of energy production by glycerol may still be the same as that by lactate, and, so long as the pyruvate produced in both cases is sufficient for the organism's needs, the rates of proliferation on the two substances may be the same.

This means that if the concentration of A in the cell diminishes to such a small value that normal proliferation with lactate will not occur it may still be sufficiently large for a normal proliferation with glycerol. It has been stated earlier that, actually, anaerobic growth of *B. coli* in a glycerol-fumarate medium takes place at a lower concentration of fumarate than in a lactate-fumarate medium. We expect therefore that the presence of —SH in the cell

[1] We are informed by Keilin that the presence of —SH seems scarcely to affect the rate of oxidation of reduced cytochrome by oxygen.

will not affect the anaerobic growth on a glycerol-fumarate medium to as great an extent as on a lactate-fumarate medium.

Experiment confirms this (Table IX).

Table IX.

To show absence of effect of a small concentration of glutathione on growth of *B. coli* in a glycerol-fumarate medium.

Conc. of glycerol × $M/20$	1	1
Conc. of fumarate × $M/20$	1	1
Conc. of glutathione × $M/250$	—	1
Relative growths after 16 hours' anaerobic incubation	2·0	2·5

PART III.

Let us now consider the effects of addition to the basal medium of a third substance—formic acid. This compound differs from succinic acid in being independent of the lactate and fumarate enzymes and from cysteine in requiring an enzyme to bring about its activation. It is specially useful for our purpose for not only is it the most powerful hydrogen donator in presence of *B. coli* (with the exception of the sugars) but it is inert as a nutritional source of carbon and its energy of oxidation is nearly that of hydrogen itself. Formates are, indeed, the most powerful source of active hydrogen so far as *B. coli* is concerned, and if the rate of proliferation of this organism is to be related to the presence in a medium of an active source of hydrogen, the inclusion of formates would be expected markedly to affect proliferation.

Anaerobic growth of *B. coli* on a lactate-fumarate-formate medium (Table X).

The effects of relatively small quantities of formates on the anaerobic growth of *B. coli* on a lactate-fumarate medium are very striking. There is a greatly accelerated growth which seems to reach its maximum when the formate concentration is about $M/40$. Above this concentration, increase of formate tends to diminish the rate of growth. Now this effect of formates is not only to be observed in a lactate-fumarate medium but in a medium composed of fumarate alone, *i.e.* with no lactate present. The growth in a fumarate-formate medium is considerably less than in a lactate-fumarate-formate medium for the same concentration of formates (see also Table XI).

Effects of formates on anaerobic growth of *B. coli* on lactate-malate and lactate-aspartate media.

It was shown by Quastel and Stephenson [1925] that anaerobic growth of *B. coli* on lactate-malate or lactate-aspartate media is doubtful or absent. Examination of the results given in Tables XII and XIII shows the powerful accelerating effects of formates on the anaerobic growth of *B. coli* in these media.

Now it seems very probable that the effect of formate is connected with its reducing activity, but to test this we have compared formate with a closely allied substance—formamide.

Table X.

To show effects of varying concentrations of formate on the anaerobic growth of *B. coli* in a lactate-fumarate medium. 17 hours' incubation. Details of all experimental technique in Part I.

Conc. of lactate × *M*/20	1	—	—	1	1	1	1	1	1	1	1	1	1·5	2	1	—	1	1	—
Conc. of fumarate × *M*/20	—	1	—	1	1	1	1	1	1	1	1	1	1	1	—	1	1	—	1
Conc. of formate × *M*/40	—	—	1	—	0·1	0·3	0·5	0·7	1	2	3	5	—	—	0·5	0·5	1	1	1
Relative growths	0	0	0	1	3	5	5	6	6	3	2	0·5	1·5	1·5	0	2	6	0	2

Table XI.

Effects of formates on anaerobic growth of *B. coli* on lactate-fumarate media giving relative growths and relative bacterial counts*. 16 hours' incubation.

Conc. of lactate × *M*/20	1	1	—	—	1	1
Conc. of fumarate × *M*/20	—	—	1	1	1	1
Conc. of formate × *M*/40	—	1	—	1	—	1
Relative growths	0	0	0	?	1	3
Relative bacterial counts	22	16	40	80	240	1080

* The bacterial counts (which were done in duplicate) were made by plating out two loopfuls of the medium diluted in sterile saline (1/200). These it must be remembered are viable counts.

Table XII.

To show effects of formates on anaerobic growth of *B. coli* on lactate-malate media. 40 hours incubation.

Conc. of lactate × *M*/20	1	1	1	—	—	1	—
Conc. of malate × *M*/20	—	1	1	1	1	1	1
Conc. of formate × *M*/40	1	—	1	—	1	2	2
Relative growths	0	0	4	0	0	4	2

Table XIII.

To show effects of formates on anaerobic growth of *B. coli* on lactate-aspartate media. 40 hours' incubation.

Conc. of lactate × *M*/20	1	1	1	—	—	—
Conc. of aspartate × *M*/20	1	1	1	1	1	1
Conc. of formate × *M*/40	—	0·5	1	—	0·5	1
Relative growths	0·5	3	3	0	?	?

Activation and effects of formamide.

Formamide at low concentrations is not a powerful donator of hydrogen. In a previous communication [Quastel, 1926] it was considered that formamide had a strong reducing activity (on methylene blue in presence of *B. coli*) even at very low concentrations. Formamide purified according to the method of Willstätter and Wirth [1909] always exhibits such activity at low concentrations. If this preparation of formamide, however, be frozen at −8°, allowed partially to thaw and the liquid portion discarded, the solid portion shows a diminished reducing activity. By repeating this operation several times a specimen of formamide may be obtained which shows but little reducing activity at low concentrations (*M*/500). At appreciably greater concentrations the formamide again becomes highly reducing, but it is probable that much of this activity is due to traces of formates still remaining in

the formamide[1]. Experiment shows that formamide does not inhibit reductions of methylene blue due to formate, *i.e.* it does not appear to be accessible to the formate enzyme.

The purest form of formamide which we were able to prepare was used in the experiments whose results are given in Table XIV. No acceleration in the anaerobic growth of *B. coli* was produced by formamide at concentrations in which formates produced powerful effects.

Table XIV.

To show relative effects of formate and formamide on anaerobic growth of *B. coli* in lactate-fumarate media. 18 hours' incubation.

Conc. of lactate × $M/20$	1	1	1	1	1	1	1	1	1	1
Conc. of fumarate × $M/40$	1	1	1	1	1	2	2	2	2	2
Conc. of formate × $M/40$	—	1	2	—	—	—	1	2	—	—
Conc. of formamide × $M/40$	—	—	—	1	2	—	—	—	1	2
Relative growths	1	3	3	1	1	1	5	5	1	1

Anaerobic growth of *B. coli* on lactate-fumarate media at varying initial hydrogen ion concentrations.

When growth occurs in a lactate-fumarate medium containing formates, the media usually become more alkaline owing to the oxidation of the formates. Grey [1914] has made use of the fact that formates are easily broken down by *B. coli* with liberation of alkali in his experiments on the fermentation of sugars. Now it seems conceivable that the accelerating action of formates noted above is due to their oxidation with resulting increased p_H and possibly therefore increased rate of proliferation.

This was tested by growing *B. coli* anaerobically in the presence and absence of formates on lactate-fumarate media, whose initial p_H ranged from 6·0 to 8·0 (Table XV).

Table XV.

To show anaerobic growth of *B. coli* on lactate-fumarate media of varying initial p_H, in the absence and presence of formates. 18 hours' incubation.

Conc. of lactate × $M/20$	1	1	1	1	1	1	1	1	1	1	1	1
Conc. of fumarate × $M/40$	2	2	2	2	2	2	2	2	2	2	2	2
Conc. of formate × $M/40$	—	1	—	1	—	1	—	1	—	1	—	1
Initial p_H	6·0	6·0	6·4	6·4	6·8	6·8	7·2	7·2	7·6	7·6	8·0	8·0
Relative growths	0	0	0·9	2·4	1	1·7	0·7	2·1	0·8	1·7	0·7	1·5

It will be seen that the anaerobic growth of *B. coli* was not accelerated by increase in p_H between the range 6·4 and 8·0. The presence of formates always accelerated the growth in this range. It seems scarcely conceivable, therefore, that the formate effect is simply an effect due to change in hydrogen ion concentration.

[1] It is worth noting that the bacterial test for formate (by reduction of methylene blue in presence of *B. coli*) is more sensitive than any chemical test for formate with which we are acquainted.

Again it should be remembered in this connection that very good growth of *B. coli* occurs anaerobically on glucose and glycerol-fumarate media; and in these media growth is always accompanied by an increase in hydrogen ion concentration[1].

Effects of formates on the anaerobic growth of *B. coli* on glucose and sodium pyruvate media.

Formates at concentrations which markedly accelerate growth in lactate-fumarate media have no perceptible action on the anaerobic growth of *B. coli* in glucose and sodium pyruvate. But relatively high concentrations of formates have a definite inhibiting action (see Table XVI).

Table XVI.

To show effects of formates on anaerobic growth of *B. coli* in glucose and pyruvate media. 18 hours' incubation.

Conc. of glucose × *M*/200	1	1	1	1	—	—	—
Conc. of pyruvate × *M*/40	—	—	—	—	1	1	1
Conc. of formate × *M*/40	—	1	2	4	—	1	4
Relative growths	4	4	4	1	4	4	1·5

Now it is well known that glucose on breakdown by *B. coli* produces formates, so that perhaps the lack of effect on the addition of small quantities of formate to a glucose medium is not surprising. On the other hand, the absence of formate action may be real, *i.e.* formates may definitely have no accelerating action. We have investigated the action of formate on glucose with the latter at a concentration of *M*/1400 and still obtained no accelerating action.

Fermentation of pyruvic acid by *B. coli*.

Investigation of the products formed from sodium pyruvate when a suspension of *B. coli* is placed in a solution of the latter shows that formates are produced in abundance. An examination of the products formed immediately after the pyruvic acid has been fermented indicates that the yield of the lower fatty acids and lactic acid does not account for all the pyruvic acid fermented. It has now been found that another substance is produced during the fermentation. This substance does not give the nitroprusside or guaiacol tests for pyruvic acid, but if a solution of it be heated in presence of a little alkali it breaks up to yield a solution once more giving the nitroprusside and guaiacol reactions. This phenomenon is being investigated further by one of us (W. R. W.).

The production of formate from pyruvate on fermentation by *B. coli* may be a reason why the former in low concentration does not accelerate the growth of *B. coli* on a pyruvate medium.

[1] Formates, as is shown later, do not perceptibly accelerate growth on a glycerol-fumarate medium. Such an acceleration would be expected if the formate effect was due to intracellular increase in p_H.

Interpretation of the formate effect.

There is little doubt that the action of formates on growth in the lactate-fumarate medium is connected with their reducing action in presence of the cell; the growth is accelerated. Yet in the case of cysteine or glutathione, where again there was little doubt that the effects were connected with the reducing action of —SH, the growth was inhibited. It appears to be quite certain therefore, as has already been concluded, that the rate of proliferation of *B. coli* cannot be directly related to the reducing or oxidising action of the medium in presence of the cell. Nevertheless formates at relatively high concentrations do inhibit growth and this inhibition is not due to toxicity as can be shown by introducing nitrates into the medium when growth is found to be accelerated. Formate shares this inhibitory action at relative high concentration with similar high concentrations of lactate, succinate or —SH, and it seems quite possible that this action is due to a high reduction potential being set up which may be neutralised by the presence of nitrates or of oxygen.

Formates are therefore abnormal in exerting a marked accelerating action at relatively low concentrations on the growth of *B. coli*. It should be possible to account for this phenomenon on the views which have been put forward to explain the results observed with succinate and with —SH.

This can quite easily be done when it is remembered (1) that the energy of oxidation of formate is nearly that of hydrogen itself,

$$H.COOH + COOH.CH:CH.COOH = CO_2 + COOH.CH_2.CH_2.COOH + 28 \text{ Cal.}$$

and (2) that formates are extremely active donators of hydrogen (in presence of *B. coli*) even at low concentrations.

It will be recalled that in interpreting the action of —SH on lactate-fumarate media and glycerol-fumarate media the suggestion was made that although the —SH brought about decreased rates of oxidation of lactate and glycerol, the energy production in the latter case was sufficiently large, but in the former case not sufficiently large, to allow the normal rate of proliferation to occur. It was assumed that sufficient pyruvate was produced for the organism's needs. The same suggestion may be made to account for the effects of formates. The formate may indeed have the effect of lowering the rate of oxidation of lactate, but, so long as this is not lowered so far that there is insufficient pyruvate produced for synthesis, the greatly increased energy production due to the oxidation of the formate itself would bring about the increased rate of growth. Thus formates should exhibit a maximum effect in their accelerating action, as is actually observed in experiment.

This explanation will account also for the accelerating action of formates on the anaerobic growth of *B. coli* on lactate-malate and lactate-aspartate media. Both malates and aspartates are hydrogen acceptors in presence of *B. coli*, though feeble compared with equal concentrations of fumarate. The fact that formates induce anaerobic growth on fumarate or malate alone is

explained when it is recalled that both fumarate and malate serve as nutritional sources of carbon in the presence of air or of nitrates. Presumably fumarate and malate can undergo self oxidation and reduction each giving rise to succinate and pyruvate (by decarboxylation of oxalacetate) and the presence of formates yield sufficient energy by their oxidation to make anaerobic growth possible. Formates will not induce anaerobic growth of *B. coli* on succinate, or lactate or glycerol or on mixtures of these substances.

This view can be put to further test. Clearly if the formate action is simply connected with the energy of oxidation of formate, the addition of a formate to a glycerol-fumarate medium should not have the same accelerating action as is observed in a lactate-fumarate medium. This is found to be the case (see Table XVII).

On these lines too we may account for the apparent absence of accelerating action of formates on growth in glucose and pyruvate media.

Table XVII.

To show the effects of formates on anaerobic growth of *B. coli* on glycerol-fumarate media. 18 hours' incubation.

Conc. of glycerol × $M/20$	1	1	1	—	—	—	1	1	1
Conc. of fumarate × $M/20$	—	—	—	1	1	1	1	1	1
Conc. of formate × $M/40$	—	0·25	1	—	0·25	1	—	0·25	1
Relative growths	0	0	0	0	1	1	3	3	3

A COMPARISON OF *B. COLI* WITH *B. SPOROGENES*.

It is interesting at this stage to compare these two organisms.

Growth of *B. sporogenes* appears to depend on the acquirement by the cell of a reduction potential of such a value that certain oxidations can be eliminated[1]. But it should be emphasised that it is not the magnitude of the potential which is of great significance; it is the fact that the substance responsible for this potential can reduce quickly enough to prevent certain oxidations (say by oxygen) bringing about a disturbance in the sequence of events which results in proliferation. If we could imagine the cysteine in a medium capable of supporting the growth of *B. sporogenes* to be entirely metal-free, we should expect no proliferation to occur in spite of the fact that metal-free cysteine has a high reduction potential [Harrison and Quastel, 1928]. The presence of —SH in a cell might prevent the oxidation by molecular oxygen of some grouping or constituent vital for the normal sequence of reactions in the cell. But this protection would depend not so much on the potential secured by the —SH as on the velocity of reduction due to the sulphydryl compound. The two, potential and velocity, are connected with each other (being both dependent on the concentration)—but the magnitude of the velocity will depend on the nature of the body catalysing the activity of the —SH.

Anaerobic growth of *B. coli* is inhibited in a lactate-fumarate medium by the presence of —SH. This we have suggested is due to the decrease in velocity

[1] Thus —SH (*e.g.* cysteine) will induce growth of *B. sporogenes* even under aerobic conditions.

of oxidation of lactate due to the presence of —SH. In effect the —SH competes with the lactate for the reduction of the fumarate. A definite velocity of oxidation of lactate—or of glycerol—is necessary for the proliferation of *B. coli*, and all the effects of succinate, —SH, and formate can be consistently interpreted on this view. The anaerobic growth of *B. coli*, as has been stated in earlier papers [Quastel, Stephenson and Whetham, 1925; Quastel and Stephenson, 1925] is not in any way fundamentally different from its aerobic growth[1].

Summary and conclusions.

We have examined the effects of three substances, succinate, cysteine (with which are associated glutathione and thioglycollate) and formate on the anaerobic growth of *B. coli*, particularly in a lactate-fumarate medium. The first substance is known to be specifically associated for its activation with the fumarate enzyme; the second is independent of the cell for its reducing action and can act in a homogeneous phase; and the third is dependent on the cell for its reducing action but is independent of the lactate and fumarate enzymes.

It is now shown that:

(*a*) Succinate inhibits the anaerobic growth of *B. coli* in the lactate-fumarate medium. The effect may be accounted for by its competition with fumarate for its enzyme but it is possible that part of the effect may be attributed to its securing a high reduction potential within the cell.

(*b*) Cysteine (or —SH) inhibits the anaerobic growth of *B. coli* in the same medium and the effect is accounted for by its inhibiting action on the velocity of oxidation of the lactate. This may be considered a true reduction potential phenomenon but it is emphasised that the effect is due rather to the speed of reduction by the —SH than to the magnitude of the reduction potential it secures.

(*c*) Formate at relatively low concentrations (when it is, however, highly reducing) increases the rate of proliferation of *B. coli* in a lactate-fumarate medium and this is accounted for by the rapid oxidation of formate with high energy yield. All the other evidence given supports these conclusions.

We may now attempt to answer the question we have set ourselves: does the growth of the cell depend on the reduction or oxidation intensity, on the r_H, of the environment? We feel justified in concluding from these experiments on succinate, —SH, and formate that the rate of growth of *B. coli* is not dependent on the oxidation or reduction intensity of its environment. Relatively high concentrations of these substances do produce, however, a similar inhibitory action on the anaerobic growth of *B. coli* and

[1] It is worth emphasising that oxygen is simply one of a number of hydrogen acceptors, each possessing its characteristic properties and each important from the point of view of cellular metabolism. It is a study of all hydrogen acceptors, which induce growth, not only that of oxygen, which is essential if a true picture of the normal life of the cell is to be obtained.

it is possible that with these concentrations certain limiting conditions are secured above which growth cannot take place. These conditions are associated probably more with kinetic considerations than with potential for it is not so much the magnitude of a reduction potential which is of significance as the activity or reducing power of the substance responsible for the potential. Cysteine, for instance, gives a high reduction potential but its reducing power (velocity of reduction) is greatly dependent on the presence of traces of metals which have little or no effect on the magnitude of its potential.

It is with the greatest pleasure that we express our gratitude to Sir F. G. Hopkins for the interest he has taken in this work.

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XVIII. PROTEIN METABOLISM IN CYSTINURIA.

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With regard to the anomaly of protein metabolism as seen in cystinuria, two schools of thought exist. On the one hand, Folin and his co-workers believe that there is but one type of the derangement, although it is capable of exhibiting varying degrees of severity. On the other hand, Neuberg considers that the condition is of three kinds, and that, whilst the presence of cystine in excessive quantities in the urine is characteristic of all its phases, yet it may be accompanied in some cases by tyrosine and leucine, and in others by putrescine and cadaverine. These very divergent views give the subject a complex aspect, and create, moreover, the difficulty of evolving a working hypothesis which might serve as a basis for the re-investigation of a problem not only of practical but of theoretical importance in that its elucidation would probably throw light on our present conceptions of normal protein metabolism. For these reasons further studies of individual cases appear necessary.

The present study arose from the admission to a ward of the Royal Infirmary, Edinburgh, of a patient with the following clinical history.

"Patient when seen was aged 23. In June, 1926, early one morning she was seized with a very acute pain in the left side of the abdomen. It was so severe that she had to get up from bed and walk about. This pain lasted for three or four hours very severely, and gradually passed off during the next two days. A good deal of vomiting was present when the pain was at its worst. Micturition caused an exacerbation of the pain. So far as she is aware she has never passed any calculi or gravel. No jaundice was present during the attack.

"Special interest was taken in the case because of the strong family history of the disease, several members having already died from the consequences of this disturbance.

"Apart from an operation for appendicitis in 1925, the patient had had no other illnesses. She was to all appearances a normal healthy girl."

Cystinuria is classified by Garrod as one of the "inborn errors of metabolism" and in this connection the reference in the above clinical notes to the occurrence of the derangement in other members of the family is highly interesting. The genealogical tree of the patient has been investigated so far as it was possible to do so, and it was found to illustrate very clearly the

hereditary aspect of the anomaly. Although there are, it is to be regretted, some gaps in the tree about which no information can be gained, nevertheless, since such records in the literature are not numerous, it is thought worthy of inclusion here (Fig. 1). The position of the subject of the present study in the family tree is indicated by the encircled figure, while crosses show the existence of members concerning whom no information is available.

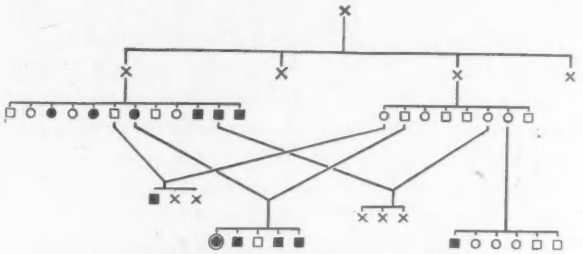

Fig. 1.

Aim of the investigation.

The original object of the present investigation was to test by urinary analysis the dietary methods advocated by various workers in this field for the treatment of cystinurics in an endeavour to ameliorate the condition of the patient in whose family several deaths, apparently attributable, directly or indirectly, to the derángement, had occurred. While the work was in progress, however, the patient displayed such keen interest in her case that the scope of the investigation was greatly extended and additional feeding experiments carried out in order to gain further information regarding the location of the disturbance. The experiments herein described may be briefly summarised as follows.

1. Hospital diet—control.
2. Hospital diet plus sodium bicarbonate.
3. ,, ,, disodium hydrogen phosphate.
4. ,, ,, cystine.
5. ,, ,, glutamic acid.
6. Low protein diet containing a minimum amount of cystine.
7. High protein diet.

Each experiment was followed by an interval during which the patient was placed on the normal hospital diet in order to stabilise her metabolism so far as possible before the next experiment was carried out.

Method.

Throughout the whole of the period of investigation the protein content of the diet was carefully controlled so that the results obtained from the analyses of the urine during the different experiments might be comparable. Even under such conditions fluctuations occur and are difficult to explain. The urine was collected under toluene in 24-hour samples and on these estimations of the total nitrogen (micro-Kjeldahl), ammonia (aeration), urea (urease), inorganic, ethereal and neutral sulphur (Fiske), amino-nitrogen (Folin) and cystine (Looney) were performed. The method of estimating the cystine requires comment. In most of the recorded investigations of cystinuria the amount of urinary cystine was determined either by its actual isolation from a concentrated urine [Gaskell, 1907] or by calculation based on the assumption that the increase either in the neutral sulphur itself [Alsberg and Folin, 1905] or in the ratio of neutral sulphur to total sulphur [Mester, 1879] was due to the excretion of cystine as such. Such methods can scarcely be regarded either as being simple or accurate. For our present purpose, however, the colorimetric method devised by Looney [1922] was available, and, since preliminary attempts at the quantitative recovery of purified cystine added to both normal and cystinuric urines gave satisfactory results, the method was used throughout the investigation. While the later stages of the work were in progress a modification by Hunter and Eagles [1927] of the original method of Looney was published. The replacement of sodium carbonate by sodium hydroxide as one of the reagents in the method certainly eliminates the possibility of "cloud" formation which occasionally occurs when the original method is used. Nevertheless the results obtained by both methods on the same solutions of cystine do not differ outside the limits of colorimetric methods of estimation.

At intervals, estimations of the non-protein-nitrogen, urea and chloride were carried out on samples of blood taken from the patient.

In addition to this quantitative work, qualitative tests on the urine, sometimes concentrated under reduced pressure, for tyrosine, leucine, cadaverine and putrescine, were performed. On numerous occasions, moreover, the patient's faeces were examined for the presence of the last-named two substances.

Analysis of results.

1. *Hospital diet.* Before the main feeding experiments were attempted it was essential to discover how far the patient's metabolism could be stabilised, especially with regard to the excretion of cystine. For this purpose the patient was kept in bed and given a convalescent diet yielding on an average 1750 calories. The results obtained during the latter end of this period, given in Table I, show that the cystine output varied considerably from day to day and bore no relation to the total nitrogen in the urine. A possible reason for

Table I.

Date (1926)	Protein in diet g.	Urine cc.	Nitrogen g.			Cystine g.			Sulphur g.			Amino-N g.	Remarks
			NH_3-	Urea-	Total	Aq.	Ppt.	Total	Inorg.	Neut.	Total		
10. xi.	69	1885	0·26	4·64	9·05	0·08	0·02	0·10	1·05	0·47	1·52	0·29	
11. xi.	75	1300	0·28	4·16	8·18	0·15	0·05	0·20	0·81	0·33	1·22	0·20	
12. xi.	75	1060	0·31	3·08	6·68	0·54	0·13	0·67	0·74	0·80	1·56	0·17	
13. xi.	69	1380	0·29	2·61	7·73	0·58	0·21	0·79	0·96	0·89	1·94	0·27	
14. xi.	66	1463	0·34	2·00	7·90	0·21	0·15	0·35	0·91	0·80	1·82	0·26	
Table II.													
6. xii.	75	1390	0·20	3·25	6·81	0·26	0·06	0·32	0·80	0·50	1·32	0·21	
7. xii.	85	1060	0·15	2·71	6·84	0·27	0·07	0·34	0·74	0·47	1·24	0·21	23·4 g. $NaHCO_3$
8. xii.	63	1830	0·23	2·34	9·15	0·56	0·09	0·65	0·96	1·06	2·04	0·27	,,
9. xii.	74	1700	0·20	3·55	8·33	0·78	0·02	0·81	1·04	0·92	1·97	0·24	,,
10. xii.	72	1400	0·20	3·58	8·54	0·38	0·15	0·53	0·99	0·65	1·66	0·22	,,
11. xii.	67	1250	0·23	3·15	7·00	0·27	0·07	0·34	0·74	0·64	1·44	0·17	
12. xii.	62	900	0·16	3·26	6·48	0·12	0·07	0·19	0·74	0·29	1·18	0·15	
13. xii.	65	800	0·26	2·94	6·36	0·23	0·05	0·28	0·65	0·34	1·13	0·16	
Table III.													
21. xi.	60	1234	0·36	3·09	7·16	0·48	0·09	0·57	0·84	0·64	1·59	0·20	
22. xi.	67	1340	0·23	3·12	7·10	0·64	0·17	0·84	0·58	0·78	1·45	0·27	
23. xi.	78	895	0·35	3·05	6·48	0·20	0·13	0·33	0·43	0·25	0·72	0·21	6 g. Na_2HPO
24. xi.	67	1240	0·36	3·36	6·95	0·36	0·15	0·51	0·47	0·47	0·99	0·20	,,
25. xi.	72	1670	0·35	3·32	8·18	0·13	0·18	0·31	0·51	0·40	0·95	0·26	,,
26. xi.	76	1140	0·30	2·09	7·18	0·35	0·07	0·42	0·36	0·50	0·88	0·27	,,
27. xi.	72	1170	0·39	2·54	6·98	0·40	0·37	0·77	0·38	0·59	0·99	0·27	
Table IV.													
15. xi.	66	1050	0·21	2·25	6·32	0·08	0·14	0·22	0·61	0·45	1·10	0·18	
16. xi.	65	1530	0·18	4·34	8·26	0·19	0·23	0·42	0·98	0·77	1·78	0·38	2 g. cystine
17. xi.	70	1200	0·23	3·84	8·52	0·18	0·37	0·56	1·90	0·70	2·70	0·37	4 ,,
18. xi.	74	1700	0·61	4·72	9·01	0·11	0·01	0·13	3·03	0·51	3·66	0·34	6 ,,
19. xi.	74	1000	0·54	5·00	9·40	0·26	0·02	0·28	3·74	1·26	5·02	0·16	8 ,,
20. xi.	74	1707	1·01	7·75	10·41	0·38	0·03	0·41	4·53	0·70	5·25	0·32	
21. xi.	60	1234	0·36	3·09	7·16	0·48	0·09	0·57	0·84	0·64	1·59	0·20	
Table V.													
27. xi.	72	1170	0·39	2·54	6·98	0·40	0·37	0·77	0·38	0·59	0·99	0·27	
28. xi.	42	1210	0·25	1·80	4·92	0·50	0·11	0·61	0·23	0·22	0·47	0·17	
29. xi.	69	785	0·32	1·88	6·20	0·08	0·18	0·27	0·64	0·33	1·00	0·18	4 g. glutamic acid
30. xi.	68	1250	0·49	4·63	9·50	0·26	0·17	0·43	0·91	0·39	1·40	0·26	6 ,,
1. xii.	59	1270	0·33	4·00	9·21	0·56	0·19	0·75	0·81	0·54	1·36	0·25	8 ,,
2. xii.	70	1460	0·31	2·54	5·78	0·27	0·04	0·31	0·77	0·40	1·27	0·29	8 ,,
3. xii.	64	1020	0·41	2·40	6·56	0·20	0·06	0·26	0·79	0·53	1·40	0·26	

this fluctuation was thought to lie in the varying quantity of cystine in the diet, and although it is impossible to eliminate entirely this amino-acid from the food, a dietary containing the minimum amount of cystine was fed during the remainder of the investigation. Reference will again be made to this point when the results of the experiments with the low and high protein diets (Exps. 6 and 7) are considered.

2. *Hospital diet plus sodium bicarbonate.* One of the methods advocated by different workers from time to time for the treatment of cystinurics has been the administration *per os* of sodium bicarbonate, the purpose being to render the urine strongly alkaline and so diminish, owing to the increased solubility of cystine, the liability of the formation of concrements in the urinary tract. Instances, however, are on record where such treatment even resulted in the actual reduction of the amount of cystine excreted. Thus Klemperer and Jacoby [1914] found that on feeding sodium bicarbonate the excretion of cystine gradually fell to zero only to recur when the use of this drug was stopped. More recently Looney *et al.* [1923] showed that the administration of 15–20 g. of sodium bicarbonate per day produced a definite fall in the amount of cystine excreted, and that this was accompanied by a marked disturbance in the distribution of the sulphur in the urine, indicating that the alkalosis produced had a definite influence on the course of protein metabolism.

As will be seen from Table II, which gives the results of one of several feeding experiments with sodium bicarbonate (23·4 g. per day), the findings of the latter workers could not be confirmed. The immediate effect was an increase in the amount of cystine excreted followed by a decrease to a figure a little lower than the original one. An increase in the neutral sulphur fraction took place though this is possibly related to the increased elimination of total nitrogen. It may be considered that the preliminary increase was due to the solution of cystine previously lodged in the urinary tract, but such an idea cannot be seriously entertained since similar rises occurred in three different experiments following one another at short intervals.

3. *Hospital diet plus disodium hydrogen phosphate.* The use of disodium hydrogen phosphate in preference to sodium bicarbonate for the purpose of producing an alkalosis is advocated by some workers, and an experiment was carried out on the lines of the preceding one with this compound. 6 g. were fed per day. On November 26, four days after the experiment started, the urine was definitely alkaline. At that point, however, there was a distinct fall in the amount of urea and total nitrogen excreted, a fact which is difficult to understand since the alkalinity produced by the sodium bicarbonate in the previous experiment was accompanied by a rise in the excretion of both of these components. Moreover so far as the excretion of cystine is concerned the use of disodium hydrogen phosphate offers no advantages over that of sodium bicarbonate. Results obtained during this experiment are given in Table III.

4. *Hospital diet plus cystine.* It has long been known that the cystinuric could oxidise a certain amount of ingested cystine to inorganic sulphate.

Little work of a quantitative character, however, has been done on this aspect of the problem. Moreover, with the view of attempting to find whether there was a peak in the ability of the patient to oxidise cystine given in this way, increasing amounts were given starting with 2 g. on November 16, and ending with 8 g. on November 19, so that a total of 20 g. of cystine were fed during the period of 4 days.

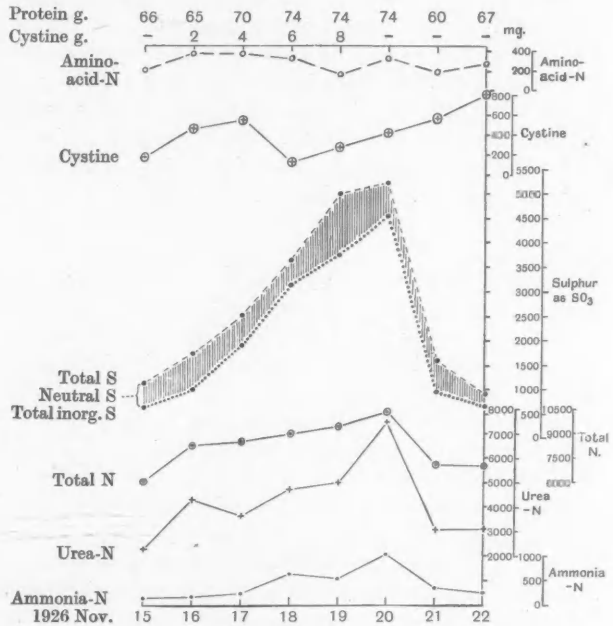

Fig. 2.

Analysis of the results obtained during the period covering this experiment (Table IV) shows that, assuming 0·60 g. (excreted on the days immediately preceding and following the period of the experiment) as the average amount of inorganic sulphate normally excreted, the additional inorganic sulphate eliminated during the feeding experiment was 11·5 g. This could only have arisen from the 20 g. of cystine fed, an amount which, theoretically, should yield 13·5 g. of inorganic sulphate. Hence some 85 % of the cystine administered must have been decomposed, a result which definitely shows that in this case at least the mechanism of the processes of oxidation of the cystine-sulphur is not at fault. Moreover, from the shape of the curve (Fig. 2) of the excretion of inorganic sulphate, it is apparent that the peak of the ability of the patient to oxidise cystine fed by the mouth had not been reached. It is to be noticed that there is no increased elimination of cystine as such during the period in question.

As will be seen from the results recorded, the increase in the inorganic sulphate in the urine is accompanied by a definite increase in the amount of ammonia excreted, a coincidence which follows from normal kidney function.

5. *Hospital diet plus glutamic acid.* One of the many peculiar features of the derangement of metabolism exhibited by cystinurics is the low ratio of urea-nitrogen to total nitrogen in the urine, and this is clearly seen from the results in the various tables to be characteristic of the patient under study. This together with the fact that there is an abnormal amount of cystine in the urine would make it appear at first sight that the processes of deamination are not functioning normally. The question then arises whether this abnormality is closely confined to the cystine molecule or is suffered generally by the whole of the amino-acids of the protein molecule. In order to study this aspect of the problem it was decided to feed a second amino-acid, glutamic, of which a small quantity was available. Following the lines adopted in the previous experiment, this was added to the diet in quantities of 4, 6, 8 and 8 g. on successive days.

From the figures recorded in Table V, it will be seen that the immediate effect was a rise in the total nitrogen and urea-nitrogen excreted, the rise in urea-nitrogen easily accounting for the additional nitrogen fed as amino-acid. It may thus be concluded that the power of general deamination is not lacking in this patient.

6 and 7. *Feeding experiments with a low protein diet* (6) *and with a high protein diet* (7). The feeding experiments with the amino-acids cystine and glutamic acid show beyond doubt that in the case under investigation the processes of oxidation and deamination are functioning normally, and that a search must be made elsewhere for an answer to the question as to what is the origin of the urinary cystine and how it escapes the normal processes of metabolism. It has already been shown (Table I) that in attempting to stabilise the patient's metabolism, it was necessary, on account of the large fluctuations in the amount of cystine in the urine from day to day, to eliminate so far as possible the cystine-rich proteins from the diet. Despite the fact that the concensus of opinion appears to favour the view that there is no connection between the diet and the amount of cystine in the urine, the results of our preliminary feeding experiments did not agree with such a conception. To gain further evidence on this point, feeding experiments were carried out in which the diet contained firstly only some 30 g. of protein and secondly 65–75 g. of the same proteins. The results of these experiments are given in Tables VI and VII respectively. That a larger amount of protein was absorbed on the high protein diet is readily seen in the higher values of the urinary total nitrogen. Accompanying this rise in the amount of protein absorbed, however, is a rise in the amount of cystine excreted from 0·71 g. (average for 9 days) to 1·1 g. (average for 9 days). This rise is large and is in almost direct ratio with the additional nitrogen eliminated, so that it is difficult to see how the conclusion that the urinary cystine is related to the protein

of the diet can be avoided. So long, therefore, as the cause of the anomaly remains unknown, it would appear that from the point of treatment cystinurics at times of crises should have their dietary protein cut down so far as is practicable, due regard being paid to the question of nitrogen equilibrium.

Table VI.

Date	Urine cc.	Nitrogen NH_3- g.	Nitrogen Urea- g.	Nitrogen Total g.	Chlorides g.	Phosphates g.	Cystine Aq. g.	Cystine Ppt. g.	Cystine Total g.	Diet
17. vi.	1050	0·159	1·395	5·25	7·5	1·198	0·495	0·105	0·600	*Low cystine*
19. vi.	1160	—	—	7·77	4·76	1·455	0·399	0·200	0·599	Protein 30 g.
20. vi.	995	0·229	3·19	5·97	3·72	1·248	0·580	0·209	0·789	Calories 1500
21. vi.	800	0·232	2·48	4·92	2·92	0·816	0·576	0·134	0·710	
22. vi.	1060	0·175	2·86	4·88	3·52	1·059	0·493	0·144	0·637	
23. vi.	1080	0·230	3·24	5·56	4·04	1·256	0·599	0·133	0·732	
24. vi.	1320	0·158	3·3	6·54	6·13	1·49	0·785	0·175	0·960	
25. vi.	1050	0·229	4·2	6·62	4·68	1·01	0·644	0·105	0·749	
26. vi.	835	0·164	2·97	5·05	3·42	1·10	0·574	0·059	0·633	

Table VII.

28. vi.	1150	0·245	4·26	7·02	6·77	1·44	0·573	0·114	0·687	*Convalescent*
29. vi.	1740	0·254	5·85	9·13	11·2	2·3	1·296	—	1·296	Protein 65/75 g.
30. vi.	1310	0·183	5·11	7·73	7·26	1·66	1·04	0·088	1·128	Calories 1900
1. vii.	1420	0·168	4·68	7·95	8·8	1·68	1·19	0·143	1·333	
2. vii.	1630	—	—	9·78	11·92	2·1	1·157	0·141	1·298	
3. vii.	700	0·147	3·01	4·52	4·50	1·03	0·544	0·189	0·733	
4. vii.	1625	0·205	6·5	9·75	11·9	1·82	1·38	0·182	1·562	
5. vii.	990	0·111	3·47	4·85	5·12	0·971	0·724	—	0·724	
6. vii.	1395	0·165	6·55	8·44	8·22	1·91	1·18	—	1·18	
7. vii.	1165	0·792	3·49	6·11	7·07	1·14	0·65	—	0·65	

Occurrence in the urine of tyrosine and leucine, and the diamines, putrescine and cadaverine. Although various workers have also reported the presence of the amino-acids, tyrosine and leucine, and the diamines, putrescine and cadaverine, yet the search for these two substances has by no means been uniformly successful. Nevertheless, the number of cases reported as showing that the excretion of abnormal quantities of cystine is only one of the manifestations of this derangement of protein metabolism was considered sufficiently large to warrant the examination of the patient's urine for the presence of these compounds. On three separate occasions during the progress of the investigation 3–5 litres of urine were concentrated under reduced pressure. In each instance the results were as follows.

(*a*) Neither leucine nor tyrosine could be isolated from the concentrate, while the colour reaction obtained with Millon's reagent was no more intense than that obtained with a similarly concentrated normal urine.

(*b*) Treatment of the concentrate with β-naphthalenesulphonic chloride according to the method of Abderhalden and Shittenhelm [1905] gave a gummy precipitate which could not be made to crystallise.

(*c*) Both the benzoylation process of Baumann and Udransky [1889] and the phenyl *iso*cyanate method of Loewy and Neuberg [1904] gave amorphous precipitates which would not crystallise after repeated attempts at purification.

It was concluded, therefore, that none of the above amino-acids or amines was present in sufficient amount to permit of the isolation of a crystalline derivative.

Blood estimations. Estimations of the blood urea-nitrogen and non-protein-nitrogen carried out at different intervals on the patient's fasting blood gave results as follows: urea-N 9·5, 10·0, 9·8 mg. per 100 cc.; N.-P.-N, 20·0, 20·5, 20·3 mg. per 100 cc. All these figures are decidedly subnormal.

Discussion.

The fact that the presence of neither the amino-acids, tyrosine and leucine, nor the diamines, putrescine and cadaverine, could be detected in the urine of the subject of the present study adds yet one more case to the growing number of cystinurics in whom the derangement of protein metabolism is limited strictly to the excretion of an abnormal quantity of cystine in the urine. Moreover, the results of the feeding experiments with the isolated amino-acid, cystine, which show that this compound is quantitatively oxidised to inorganic sulphate, confirm the findings of earlier workers in this field, *e.g.* Hele [1909] and Looney *et al.* [1923], and support the conclusion that the cystinuric oxidises cystine fed as such by the mouth in a perfectly normal manner. Such a conclusion stands in strong contradistinction to the statement of Lewis [1924] that "the failure of the organism to oxidise one specific amino-acid in cystinuria would support the theory that the processes of deamination and oxidation are not the same for all the constituent amino-acids of the protein molecule," for which little, if any, evidence exists at the present time. Moreover, the fact that the peak of the curve of the ability of the patient to oxidise cystine had not been reached, even when so much as 8 g. per day were administered, makes it difficult to understand the suggestion of Hunter and Eagles [1927] that "one of the errors of cystinuria lies in the incapacity of the liver to handle the cystine normally in reserve there." That the cystinuric can oxidise large quantities of cystine administered orally, together with the results of the final feeding experiments showing that, contrary to general opinion, the quantity of urinary cystine varies directly with the amount of protein fed, would make it appear that "the error" is not one connected either with the normal process of oxidation or with the organ in which that process takes place.

Rather would it appear that the disturbance is one closely related either to the digestive processes of the organism or to the permeability of the intestinal wall. The fact that cystine fed as such is completely oxidised while some of the cystine arising from dietary protein escapes oxidation would suggest that the amino-acid in the latter case is actually passing from the intestinal tract into the portal system, not in the isolated form, as it does normally, but as a peptide, and as such escapes the normal oxidative processes of the liver. Fission of the peptide might result when it reached the tissues

and encountered the intracellular enzymes. Cystine would then be liberated only to be excreted by the kidney owing to its presence in abnormal amount in the blood-stream.

Such an idea receives other experimental support apart from that included in this paper. Blum [1903] found that experimental cystinuria could be produced by the injection of cystine into the peripheral circulation but not when it was injected into the mesenteric venous circulation. Such facts would be met if, in the cystinuric, the passage of the liver was effected by the cystine in the form of a peptide, in which it was so linked up that it escaped the normal processes of deamination and oxidation.

A possible solution of the problem of metabolism presented by the cystinuric lies in the feeding of complex peptides containing cystine, and it is probable that a more exact knowledge of the action of enzymes on the protein molecule might give a clearer indication of the source of the urinary cystine in this type of condition.

As regards the more practical question of the treatment of cystinurics, it would appear that the only safe method is that of cutting down the dietary protein of the patient so far as is consistent with the maintenance of nitrogen equilibrium. From the results obtained there is nothing to recommend the continual use of sodium bicarbonate.

Summary.

Evidence is advanced that the cystinuric oxidises cystine to inorganic sulphate normally. Neither the amino-acids, tyrosine and leucine, nor the diamines, putrescine and cadaverine, could be found in the urine of the patient who formed the subject of the present study.

The amount of cystine in the urine appears to bear some relation to the quantity of dietary protein.

The administration of sodium bicarbonate, but not of disodium hydrogen phosphate, to the cystinuric is accompanied by a marked disturbance of the sulphur fractions of the urine. Neither of these compounds affects the excretion of cystine to any extent.

In reporting this work, the author desires to take this opportunity of expressing his deep indebtedness to Dr Robert Thin of Edinburgh for facilitating the carrying out of this research, and also for providing the exceedingly useful information regarding the genealogical tree of the subject. The work was carried out with the help of a grant from the Medical Research Council which is hereby acknowledged.

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XIX. A STUDY OF FACTORS SAID TO INFLUENCE THE NITROGEN DISTRIBUTION OF GELATIN.

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WITHIN the past few years, several publications have appeared from the Biochemical Department of the Imperial College of Science and Technology, London, concerning the effect on the nitrogen distribution of gelatin of certain preliminary treatments of the protein. In discussing these publications and in describing experimental procedures, terminologies which have been well established by custom will be employed. By the percentage of a type of nitrogen is indicated that part of the total nitrogen of the protein which, in the hydrolysate or in a designated fraction of it, appears in this form; the basic nitrogen refers to the nitrogen of those compounds which are precipitated, under the defined conditions, by phosphotungstic acid.

According to Knaggs [1923], if gelatin is allowed, prior to the hydrolysis proper, to stand at laboratory temperature with 20 % HCl, a marked increase in the percentage of basic nitrogen takes place; the percentage of amino-nitrogen is, however, unchanged. He attributes the increase in the basic nitrogen to the formation of difficultly hydrolysable polypeptides.

Knaggs and Schryver [1924] report that while the percentage of basic nitrogen remains constant, the percentage of non-amino-nitrogen of the filtrate is increased by certain treatments of this protein. In their experiments, before hydrolysis proper, gelatin was allowed to stand at laboratory temperature with *N* HCl or *N* NaOH or was heated at 105° with water. The effect of the three treatments on the nitrogen distribution of the protein is said by the authors to be, in general, the same. In regard to the treatment with water, the percentage of non-amino-nitrogen of the filtrate is shown to increase gradually with the time of heating until it reaches a fairly constant maximum.

These two sets of results appear to be somewhat inconsistent. Moreover, the results of Thornley [1927], coming from the same laboratory, are in serious disagreement with both of them. On the authority of Thornley's experimental results and the curve that he constructed from them, the preliminary treatment of the protein with *N* HCl or *N* NaOH causes a large increase in the percentage of basic nitrogen; preliminary treatment with 20 % HCl, on the other hand, causes a smaller increase in this fraction. The former findings are contrary to

those of Knaggs and Schryver, the latter are contrary to those of Knaggs. In agreement with Knaggs and Schryver, however, Thornley finds that heating gelatin with water causes practically no change in the basic nitrogen fraction. He finds, further, that subsequent treatment of the same material with HCl does not cause an increase of this percentage. Figures for non-amino-nitrogen are not included in his published results.

Finally, Schryver and Buston [1927] attribute the increase in the percentage of basic nitrogen, which they observed, to *dl*-lysine. The details of their isolation of this amino-acid are reported.

In many cases, we are unable to deduce from these publications the number of duplicates which have contributed to the results and the degree of agreement of these duplicates. There are, in addition, various inconsistencies in the conclusions drawn from the experimental work. The investigators agree, however, that the nitrogen distribution of gelatin is altered by preliminary treatment of the protein with acid or alkali. Were this fully established, it would be necessary to conclude that the protein molecule is much more labile than has been hitherto supposed. The figures appearing in the literature as nitrogen distribution numbers could not, moreover, be regarded as definite for the proteins in question. In view of the importance of these data, Professor Sørensen suggested to the writer that an investigation of the subject be carried out. The results obtained by this investigation show no change in nitrogen distribution due to preliminary treatment of gelatin with acid or alkali, and fail, accordingly, to support the principal contention of the investigators mentioned.

Experimental.

In order to become thoroughly familiar with the methods of analysis and to investigate certain parts of the procedure, we carried out twenty preliminary nitrogen distribution experiments. The samples of gelatin used for these analyses were divided into groups on the basis of the treatment given them prior to the hydrolysis proper. Of the results, only those which pointed the way to the experimental method adopted are mentioned. The nitrogen distribution numbers recorded in Table IV under their group headings (A, B, C and D) are derived from twelve later analyses of gelatin. The details of the method used for obtaining and classifying them are given below. The conclusions to be drawn from the two sets of experiments are the same but the earlier ones were carried out on smaller amounts of material.

Treatment and hydrolysis of gelatin.

The different treatments with acid and alkali accorded to gelatin by Knaggs and by Schryver were duplicated as closely as was compatible with obtaining uniformity of samples. To obtain the desired uniformity, the protein was dissolved in hot water, care being taken to obtain a homogeneous solution. The ratio by weight of gelatin to water was approximately 11 : 100. 100 cc.

portions of the hot solution were pipetted into flasks and weighed. Upon the cooling of the material, gels formed.

The total nitrogen per gram of solution was determined on a separate sample. 20 cc. were diluted to a volume of 500 cc., and portions of the dilute solution were analysed. Here, and wherever possible in subsequent steps of the procedure, solutions and the parts of them used for analysis were weighed. Where this was not feasible, a method of calibration was employed.

The preliminary treatments of the 100 cc. samples are described in the following paragraphs. The concentration of HCl in each of the solutions was brought to 20 % before the hydrolysis proper of the gelatin, which was accomplished by boiling the acid solution for 24 hours. Hang-in reflux condensers were used.

Group A. No preliminary treatment. The gel was liquefied by heat and the resulting solution heated almost to boiling. A hot solution of HCl was added and the gelatin immediately hydrolysed.

Group B. Treatment with N *NaOH.* The gel was liquefied by heat and a solution, containing the calculated amount of NaOH, was added; the flask was thoroughly shaken and at once plunged into a cooling-bath. The mixture was allowed to stand for 24 hours at room temperature. At the expiration of this time, a hot solution of HCl was added and the gelatin was at once hydrolysed.

Group C. Treatment with N *HCl.* The gel was liquefied by heat and a solution containing the calculated amount of HCl was added. The procedure given for Group B was then followed except that the time of standing was 48 hours.

Group D. Treatment with 20 % *HCl.* The gel was liquefied by heat, a solution of HCl of proper strength was added and the flask was plunged into a cooling-bath. As soon as the mixture had stood for 24 hours at room temperature, the gelatin was hydrolysed.

Group A, then, consisted of controls, approximately 10 g. of protein being hydrolysed by some 25 times its weight of 20 % HCl. The members of Group B differed in that, prior to the hydrolysis proper, they were allowed to stand for 24 hours with *N* NaOH and in that the hydrolysis was accomplished in the presence of NaCl. The members of Group C differed from the controls in that they were allowed to stand for 48 hours with *N* HCl, and the members of Group D in that they were allowed to stand for 24 hours with the acid used for hydrolysis.

Comparison with the publications of Knaggs and of Knaggs and Schryver will show the similarity of the experimental conditions. The chief point of difference is that, in our experiments, the gelatin was first heated for a short time with water. According to Knaggs and Schryver, any noteworthy change in the protein, due to this treatment with water, would make itself apparent by an increase in the percentage of the non-amino-nitrogen of the filtrate. It is extremely unlikely that such an increase would be compensated by

a corresponding decrease in the non-amino-nitrogen of the precipitate, particularly since the total nitrogen of the latter fraction was reported by Knaggs and Schryver as remaining unchanged. A change in the protein should, consequently, be accompanied by an increase in the percentage of total non-amino-nitrogen.

To make certain that such a change had not occurred, a control determination was carried out. Gelatin was dropped into boiling HCl and hydrolysed and the ammonia and humin were removed from the hydrolysate. Portions of the residue were used for determinations of total nitrogen and of amino-nitrogen. By the formaldehyde titration, 17·81 % and by the nitrous acid method, 27·80 % of the total nitrogen of the hydrolysate was in the non-amino-form in this residue. Corresponding figures for the members of Group A average 17·82 % and 26·97 % respectively. From the non-amino-nitrogen test of two of the above investigators, then, it would seem that our treatment with water did not greatly alter the protein material. Our preliminary treatments of gelatin should, consequently, be justly comparable to theirs.

To make sure that no errors would arise from incomplete hydrolysis of the protein, the method adopted was compared with hydrolysis for 1½ hours in the autoclave at approximately 150°. Samples hydrolysed in the autoclave gave no greater figures for amino-nitrogen but higher figures for ammonia-nitrogen than those hydrolysed at ordinary pressures. Hydrolysis at ordinary pressures for 24 hours was, therefore, deemed sufficient. Since the figures for ammonia-nitrogen obtained by these experiments are needed for a part of the discussion in the next section, they are given in Table I.

Table I.

Hydrolysis	% of total N as ammonia-N
Ordinary pressure: 20 % HCl	1·49 (average of 8 determinations)
Autoclave: 10 % HCl	2·27 (average of 6 determinations)
Autoclave: 20 % HCl	2·81 (average of 6 determinations)

Maximum deviation of an individual figure from any average: 0·36 % of total nitrogen.

Determination of ammonia-nitrogen.

For the removal of ammonia, MgO and boiling at ordinary pressures, CaO and boiling at low pressures, and NaOH and aeration have been used by different investigators. The first and second of these methods, however, have been severely criticised. As noted by Jodidi and Moulton [1919], MgO, in excess of that required to release the ammonia and saturate the solution, occludes some nitrogen-containing substances other than those correctly given the name of humin. The amount of nitrogen occluded is roughly proportional to the amount of excess MgO and the substances are not removed by washing the oxide with hot water. Our results confirm this observation. The larger the amounts of MgO used, the higher are the apparent values for humin-nitrogen. Although it has not been tested, it is likely that CaO as well causes

an error at this point. Treatment of the excess oxide with HCl before filtering should, by giving soluble products, eliminate this error.

The MgO and CaO procedures, as originally presented, must be criticised on another count. As will appear in the section on precipitation with phosphotungstic acid, the amount of inorganic salt introduced into the solution of amino-acids should be known. The original oxide methods are unsatisfactory in this respect. Thimann [1926] gives a thorough discussion of this point. Although his conclusions are to the contrary, it seems to us that this offers a second important reason for treating the excess oxide with HCl. If a known amount of oxide is added to the hydrolysate and this oxide is completely changed to the soluble chloride, it is self-evident that the amount of salt introduced into the solution can quickly be calculated.

Owing to the fact that cystine is not present in the hydrolysate from gelatin, the question of its decomposition by heating to 100° in alkaline solution need not be considered. Either of the oxide methods, altered as indicated, should be adequate to the purpose at hand. For this particular investigation, however, it was not judged advisable to make use of either of them. NaCl was earlier introduced into the members of Group B by the preliminary treatment of the protein samples with NaOH and the subsequent acidification. Complication of matters by the introduction of a second salt was not desired. This consideration limited us to a procedure involving the use of NaOH.

In the MgO method, the hydrolysate is heated to 100° in alkaline solution. Such a practice should be as permissible in any other method, provided that the p_H of the solution be no higher, a factor which may be controlled by the use of the proper indicator. The procedure finally adopted entails the use of NaOH and boiling at ordinary pressures, the p_H being controlled by titration of the solution with phenolphthalein as the indicator. Before its adoption, it was compared to the MgO method. The results of this comparison are given in Table II. From them, it may be seen that the amounts of ammonia obtained by the two methods do not greatly differ and that the addition of several millimols of NaOH in excess of the proper amount does not markedly affect the results.

Table II.

	% of total N as ammonia-N (average of duplicates)
MgO (g.)	
3	1·32
4	1·35
1·6 *N* NaOH (cc.)	
54	1·32
55	1·34
56	1·36
57	1·36
60	1·39
65	1·40

Maximum deviation from any average: 0·02 % of total nitrogen.

The method was applied as follows. A hydrolysate, after being twice concentrated to small volume on the steam-bath to remove HCl, was introduced into a 1·5 litre flat-bottomed flask and a few drops of phenolphthalein were added. The solution was then titrated with NaOH to a distinct pink. Additional NaOH, sufficient in amount to react with the ammonium chloride present, was added and the ammonia boiled off. Immediately after the removal of the ammonia, the solution was acidified with HCl, a procedure designed to stop any decomposition of amino-acids. It is unlikely, however, that with either this or the MgO method there is a complete absence of amino-acid decomposition. In a series of eight determinations with MgO, water was added to the residue from the usual distillation and a second distillate was collected and analysed. The ammonia-nitrogen, obtained from the second distillation, averaged 0·07 % of the total nitrogen of the protein. Although these results imply that the treatment is too drastic, the error indicated is small when compared to the total error of the determination.

As may be seen from Tables I and IV, the figures obtained for the ammonia-nitrogen of analogous solutions vary greatly. On the other hand, duplicate analyses of a single solution, as given in Table II, show no such variation. From Table I, it may further be seen that different conditions of hydrolysis give rise to ammonia-nitrogen values of different magnitude. This was remarked for a number of proteins by Henderson [1899] and for gelatin by Denis [1910]. These observations, taken together, suggest that a lack of constancy of the ammonia-nitrogen figures in duplicate nitrogen distribution experiments is due to differences at present uncontrollable in the conditions of hydrolysis of the protein samples.

Determination of humin-nitrogen.

The residue, after the distillation of ammonia and the subsequent acidification, was filtered into a 250 cc. volumetric flask and the filter-paper was thoroughly washed. The precipitate was analysed and the result recorded as humin-nitrogen.

The filtrate was made up to a volume of 250 cc. and parts of it were used for determinations of amino-nitrogen and of total nitrogen and for the precipitation of the bases with phosphotungstic acid; one 5 cc. portion was titrated to neutrality. The figure obtained by this titration is of value in adjusting any sample to a definite acid concentration. This adjustment is necessary before the decolorisation for the formaldehyde titration and in the preparation of the solution for the precipitation of the bases.

Determination of amino-nitrogen[1].

By the nitrous acid method. This was carried out on 5 cc. samples according to the method of Van Slyke [1912], except that the sample was introduced

[1] When this investigation was carried out (spring 1927), the method of Linderstrøm-Lang [1927, 1928] was not elaborated.

by means of a calibrated pipette as recommended by Plimmer [1924]. The temperature was always 20° or more, so a reaction time of 30 minutes was allowed.

By the formaldehyde titration. This was carried out according to the directions of Jessen-Hansen [1923], with a modification, used in the Carlsberg Laboratory and involving the substitution of bromothymol blue for litmus paper as the indicator in the neutralisation. The use of separate samples for neutralisation and for titration with formaldehyde is thus necessitated.

A 70 cc. portion of the solution of amino-acids was used for the decolorisation and, of the resulting 100 cc., 40 cc. were used for the treatment to remove CO_2. Of the 50 cc. of CO_2-free filtrate, two 20 cc. portions were used for the analysis itself.

Precipitation with phosphotungstic acid.

Thimann [1926] reported that the precipitation of the bases with phosphotungstic acid is influenced by the concentrations of salts and of the mineral acid. Our results confirm these observations but were obtained under somewhat different experimental conditions and are, consequently, of a different magnitude. The results of these control experiments are given in Table III.

Table III.

Approximate molarity in			N (mg.) in precipitate (average of duplicates)
NaCl	$MgCl_2$	HCl	
0·01	—	1·2	13·6
0·44	—	1·2	15·2
0·87	—	1·2	16·5
0·01	0·25	1·2	14·7
0·01	0·50	1·2	15·5
0·01	—	2·3	14·4

Total nitrogen of each sample: 87·2 mg.

For the basic figures of two series of nitrogen distribution numbers to be strictly comparable to each other, then, the concentrations of acid and of salts in the solutions used for precipitation with phosphotungstic acid must be the same. The concentration of acid may easily be controlled by the determination of the acidity of each solution and by the addition of an amount of acid sufficient to bring the total concentration to the required figure. Control of the salt concentration may be accomplished by the use for hydrolysis of protein samples of equal or of known salt content, by ascertaining the amounts of salt introduced into the solution at any point in the procedure, and by a final adjustment of each solution to a definite salt concentration. The magnitude of the fluctuations in the basic fraction to be expected from a failure to control the concentrations of acid and of salt may be calculated from the figures in Table III. For this investigation, they would have been perceptible. The concentrations of NaCl in the 200 cc. precipitating solutions, arising from the NaOH used for boiling off the ammonia, varied from 0·33 *N* to 0·38 *N*. The acidity of the solutions varied from 0·07 *N* to 0·14 *N*.

The concentration of nitrogen, also, in each of the solutions used for precipitation of the bases should be kept at a constant value. This is amply demonstrated by the work of Kernot and Knaggs [1928], which is well supported by theoretical considerations. Van Slyke [1911] recognised that the phosphotungstates of the bases have solubilities great enough to be of importance. These solubilities have not been determined in the presence of known amounts of salts.

Lastly, the solution in which the precipitation of phosphotungstates is taking place should be kept at a definite temperature and a definite time should be allowed for the precipitation. A variation in the solubility of phosphotungstates with temperature was reported by Gortner and Hoffman [1925] and this variation was confirmed by Kernot and Knaggs [1928]. Our results, also, afford a confirmation. Two filtrates from 48-hour precipitations at 18° were allowed to stand an additional 48 hours at 4°. Additional precipitates, the nitrogen of which averaged 2·45 % of the total nitrogen of the protein, were obtained. Plimmer and Rosedale [1925] reported that the percentage of basic nitrogen is influenced by the time allowed for the precipitation to take place.

For our experiments, the volume in which each precipitation was allowed to take place was 200 cc., the concentrations of HCl and NaCl were 1·0 *N* and 0·75 *N* respectively; and the nitrogen in the solution before precipitation was 900–908 mg. Forty-eight hours were allowed for each precipitation to take place and the temperature of the solutions during this period was 18°.

In preparation for this precipitation, 150 cc. of the solution of amino-acids were introduced into a 300 cc. Erlenmeyer flask and the calculated amounts of HCl, NaCl and water were added. The solution, the volume of which at this point was 190 cc., was now heated almost to boiling and 15 g. of phosphotungstic acid in 10 cc. of solution were added. The flask was then allowed to stand for 48 hours in a thermostat at 18°. The precipitate of phosphotungstates appeared only after the solution began to cool. If the precipitate was allowed to form in the cold, it was found almost impossible to redissolve it by heating. Further, its manipulation was difficult and the results were erratic.

Filtration and washing of precipitate. The precipitate was collected and washed according to the method of Van Slyke [1915], except that the amount of solution used for washing was carefully controlled, as recommended by Plimmer and Rosedale [1925]. For the amount of precipitate with which we were dealing, five 20 cc. portions of the wash liquid recommended by Van Slyke, cooled to about 0°, appeared to be satisfactory, and this amount was accordingly adopted as a standard.

Treatment of the precipitate. The precipitate was dissolved in approximately *N* NaOH as directed by Plimmer and Rosedale. Of the resulting 100 cc., 10 or 20 cc. portions were used for the determination of total nitrogen. The remainder was used for the determination of amino-nitrogen by one of the methods mentioned above. For the nitrous acid method, 10 cc. samples were

used. For formaldehyde titrations it was necessary to remove the phosphotungstic acid and to make sure that the solution was free from CO_2. From 60 to 80 cc. the phosphotungstic acid was removed by the $BaCl_2$ method of Van Slyke [1911], after which the solution was acidified and evaporated to a small volume on the steam-bath. It was then given the treatment outlined by Jessen-Hansen for the removal of CO_2, by which the last traces of phosphotungstic acid also were removed. Finally, the solution was filtered into a 50 cc. volumetric flask and two 20 cc. portions were used for the analysis.

Treatment of the filtrate. The filtrate and washings were transferred to a 500 cc. volumetric flask and diluted to volume. 20 cc. samples were used for the determination of total nitrogen. From 250 cc. phosphotungstic acid and CO_2 were removed in the same manner as above and the solution of amino-acids was filtered into a 100 cc. volumetric flask. Of this, two 20 cc. portions were used for the formaldehyde titration and 5 cc. samples were used for the nitrous acid treatment.

Discussion of results.

The results of these experiments are given in Table IV. From them, it can hardly be concluded that preliminary treatment of gelatin with acid or alkali affects the nitrogen distribution. The possible exception is that the increase in the percentage of ammonia-nitrogen observable in the members of Group B may be due to the preliminary treatment of the protein with alkali. That this may not be the correct interpretation, however, is indicated by the statements of Hart [1901] and Kossel and Patten [1903] that the percentage of ammonia-nitrogen is increased by the addition of NaCl to the hydrolysing acid.

As has been stated, the solutions and the parts of them used for the various analyses were, whenever possible, weighed. Since no attempt was made to use exactly aliquot portions, it might be misleading to record the actual values of the determinations. The approximate magnitude of each analysis may easily be calculated from the figures for total nitrogen and the records of volume scattered throughout the discussion.

It is worthy of note that the values for total amino-nitrogen obtained by direct analysis are considerably higher than those obtained by adding the values for the amino-nitrogen of the precipitate to the figures for the amino-nitrogen of the filtrate. In the case of the nitrous acid method it is not difficult to assign the reason for this anomaly. Van Slyke [1911] advocates a reaction time of 10 minutes when lysine is absent from the solution to be tested and a reaction time of 30 minutes when lysine is present. This advice was followed. The filtrate from the bases was thus allowed a reaction time of 10 minutes while the other solutions were each allowed 30 minutes. In preliminary experiments it was found that four filtrates, which gave an average of 60·9 % amino-nitrogen for 10 minutes' reaction, gave an average of 65·8 %

Table IV.

A comparison of the nitrogen distribution numbers of treated and untreated gelatin.

Group	Total N found mg.	NH_2-N found (*a*) %	Humin-N found (*b*) %	Total amino-N found: Form. titration %	Total amino-N found: HNO_2 method %	Total non-amino-N calculated: Form. titration %	Total non-amino-N calculated: HNO_2 method %	Total amino-acid-N found (*c*) %	Total N (*a*)+(*b*)+(*c*) %
A	1529	1·60	0·06	80·57	71·45	18·11	27·23	98·68	100·34
A	1532	1·63	0·05	80·34	71·71	18·15	26·78	98·49	100·17
A	1532	1·39	0·04	81·45	71·75	17·22	26·92	98·67	100·10
B	1529	1·83	0·06	80·79	72·55	17·82	26·06	98·61	100·50
B	1532	1·68	0·05	80·92	71·18	17·52	27·26	98·44	100·17
B	1532	1·65	0·05	81·19	71·24	16·79	26·74	97·98	99·68
C	1530	1·58	0·05	80·05	71·45	18·50	27·10	98·55	100·18
C	1530	1·62	0·05	80·50	72·16	17·96	26·30	98·46	100·13
C	1531	1·52	0·04	80·92	71·84	17·71	26·79	98·63	100·19
D	1530	1·54	0·04	80·74	71·17	17·89	27·46	98·63	100·21
D	1530	1·78	0·07	79·98	71·33	18·19	26·84	98·17	100·02
D	1532	1·31	0·05	80·88	71·95	17·75	26·98	98·63	99·99
Av. A	—	1·54	0·05	80·79	71·64	17·82	26·97	98·61	100·20
Av. B	—	1·72	0·05	80·97	71·66	17·37	26·68	98·34	100·11
Av. C	—	1·57	0·05	80·49	71·82	18·06	26·73	98·55	100·17
Av. D	—	1·54	0·05	80·53	71·48	17·95	27·00	98·48	100·07
Av. of all	—	1·59	0·05	80·70	71·65	17·80	26·85	98·50	100·14

Amino-N of filtrate found: Form. titration %	Amino-N of filtrate found: HNO_2 method %	Non-amino-N of filtrate calculated: Form. titration %	Non-amino-N of filtrate calculated: HNO_2 method %	Total N of filtrate found (*d*) %	Amino-N of precipitate found: Form. titration %	Amino-N of precipitate found: HNO_2 method %	Non-amino-N of precipitate calculated: Form. titration %	Non-amino-N of precipitate calculated: HNO_2 method %	Total N of precipitate found (*e*) %	Total N (*a*)+(*b*)+(*d*)+(*e*) %
69·52	58·46	12·26	23·32	81·78	—	8·00	—	8·66	16·66	100·10
69·85	58·76	12·37	23·46	82·22	8·07	—	8·45	—	16·52	100·42
70·99	59·34	11·26	22·91	82·25	—	—	—	—	16·14	99·82
68·63	57·87	13·12	23·88	81·75	—	7·74	—	8·75	16·49	100·13
70·53	58·80	10·80	22·53	81·33	7·93	—	8·73	—	16·66	99·72
71·49	59·73	10·32	22·08	81·81	—	—	—	—	16·43	99·94
70·11	57·93	11·31	23·49	81·42	—	7·89	—	8·98	16·87	99·92
69·11	58·03	12·54	23·62	81·65	7·96	—	8·59	—	16·55	99·87
70·61	58·34	11·38	23·65	81·99	—	—	—	—	16·22	99·77
70·62	58·65	11·44	23·41	82·06	—	7·95	—	8·40	16·35	99·99
69·35	58·06	12·44	23·73	81·79	7·87	—	8·54	—	16·41	100·05
70·68	59·15	11·20	22·73	81·88	—	—	—	—	16·52	99·76
70·12	58·85	11·96	23·23	82·08	—	—	—	—	16·44	100·11
70·22	58·80	11·41	22·83	81·63	—	—	—	—	16·53	99·93
69·94	58·10	11·75	23·59	81·69	—	—	—	—	16·55	99·86
70·22	58·62	11·69	23·29	81·91	—	—	—	—	16·43	99·93
70·13	58·59	11·70	23·24	81·83	—	—	—	—	16·49	99·96

amino-nitrogen for 30 minutes' reaction. This accounts for the greater part of the discrepancy. The temperature at which these reactions were carried out varied from 20° to 23°.

An attempt was made to discover the cause of the loss of formaldehyde-titratable nitrogen. Since there was no loss in total nitrogen, this must have occurred in preparing the solutions for the titration. In experiments carried through for this purpose, all precipitates arising from the addition of $BaCl_2$ to the solutions containing phosphotungstic acid were analysed for nitrogen. Despite a thorough washing of these precipitates, the analyses invariably showed the presence of considerable and nearly constant amounts of nitrogen. The amounts were higher in the precipitates from the mono-amino-fraction than

in those from the basic fraction, probably owing to the larger part of the phosphotungstic acid being associated with the first fraction. To the solutions from which the phosphotungstic acid had been removed, additional amounts of this acid were added and the $BaCl_2$ treatment was repeated. Additional amounts of nitrogen were removed from the solutions by this treatment. When the precipitation of 7·5 g. of phosphotungstic acid was carried out in a volume of approximately 800 cc. an amount of nitrogen, roughly corresponding to 1·5 % of the nitrogen present in the solution, was removed. This held true whether the solution in question was one of basic amino-acids or one of mono-amino-acids. Although insufficient data have been accumulated to permit of any definite statement, the indications are that a considerable loss of nitrogen is unavoidable when phosphotungstic acid is precipitated from a solution of amino-acids by means of $BaCl_2$ and that this phenomenon is not a selective one but depends on the concentrations of the amino-acids and of the phosphotungstic acid in the solution.

For future work, the more recent amyl alcohol-ether method of Van Slyke [1915] will be used for the removal of phosphotungstic acid. From test experiments carried out on gelatin, the separation of the amino-acids from phosphotungstic acid by this method seems to be very nearly quantitative. A small amount of the latter acid remains with the amino-acids and is precipitated upon treatment of the solution with $BaCl_2$ for the removal of CO_2. However, the amount is so small that less than 0·1 % of the total nitrogen is removed at this point.

Summary.

1. The experimental results force us to the conclusion that, within the limits of accuracy of the method described, preliminary treatment of gelatin with acid or alkali has no effect on the percentages of non-amino-nitrogen and of basic nitrogen.

2. Several other factors have been reported by various workers as influencing the figures obtained as nitrogen distribution numbers of proteins. A study of several of these factors has been made. From the results of this study, certain modifications of the method of analysis of proteins are advocated. It is believed that the numbers obtained by the modified method will be, within certain limits of experimental error, fairly comparable to each other. The following paragraph gives a brief summary of the procedure as altered, particular stress being laid on the alterations.

The protein is hydrolysed by boiling with 20 % HCl. After removal of as much of the HCl as is possible by evaporation, the hydrolysate is rendered alkaline by the addition of a known amount of MgO, CaO or NaOH and the ammonia is removed under the proper conditions. The residue is acidified and the humin is filtered off. The filtrate, of which the acidity is determined, is divided into aliquot portions; some of these are used for determinations of amino-nitrogen and some for total nitrogen. In one portion the amino-acids

Total N (a)+(b)+(c) %
100·34
100·17
100·10
100·50
100·17
99·68
100·18
100·13
100·19
100·21
100·02
99·99
100·20
100·11
100·17
100·07
100·14

Total N (a)+(b)+(d)+(e) %
100·10
100·42
99·82
100·13
99·72
99·94
99·92
99·87
99·77
99·99
100·05
99·76
100·11
99·93
99·86
99·93
99·96

are divided into two groups by a precipitation with phosphotungstic acid. The concentrations of salt or of salts, of acid and of nitrogen are always brought to definite values in this latter portion before the precipitation is allowed to take place. The solution of amino-acids is heated almost to boiling before the phosphotungstic acid is added, and the resulting mixture is kept at a definite temperature during the definite time allowed for precipitation. The phosphotungstates are filtered off and are washed according to a rigidly defined technique. If determinations of the amino-nitrogen of the filtrate and of the precipitate are to be made by the nitrous acid method only, no preparation of the filtrate is necessary and the precipitate may be obtained in a suitable form by its solution in *N* NaOH. Should it be desirable to make use of the formaldehyde or alcohol titrations, the phosphotungstic acid is removed from each of the amino-acid fractions by the amyl alcohol-ether method. The amino-nitrogen and the total nitrogen of the filtrate and of the precipitate are determined.

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